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7) Title: APPARATUS AND METHOD FOR DETECTING AND IDENTIFYING INFECTIOUS AGENTS			
8) Abstract <p>Solid phase methods for the identification of an analyte in a biological medium, such as a body fluid, using bioluminescence are provided. A chip designed for performing the method and detecting the bioluminescence is also provided. Methods employing biomaterialization for depositing silicon on a matrix support are also provided. A synthetic synapse is also provided.</p>			

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APPARATUS AND METHOD FOR DETECTING AND IDENTIFYING INFECTIOUS AGENTS

RELATED APPLICATIONS

This application claims priority to U.S Provisional application Serial

5 No. 60/037,675, filed February 11, 1997 and to U.S. Provisional application Serial No. 60/033,745, filed December 12, 1996.

Certain subject matter in this application is related to subject matter in U.S. application Serial No. 08/757,046, filed November 25, 1996, to Bruce Bryan entitled "BIOLUMINESCENT NOVELTY ITEMS" (B), and to U.S.

10 application Serial No. 08/597,274, filed February 6, 1996, to Bruce Bryan, entitled "BIOLUMINESCENT NOVELTY ITEMS". This application is also related to U.S. application Serial No. 08/908,909, filed August 8, 1997, to Bruce Bryan entitled "DETECTION AND VISUALIZATION OF NEOPLASMS AND OTHER TISSUES" and to U.S. Provisional application Serial No. 15 60/023,374, filed August 8, 1996, entitled "DETECTION AND VISUALIZATION OF NEOPLASMS AND OTHER TISSUES", and also to published International PCT application No. WO 9?/ . .

Where permitted, the subject matter of each of the above noted U.S. applications, provisional applications and International application is herein 20 incorporated by reference in its entirety.

FIELD OF INVENTION

The present invention relates to methods for the identification of an analyte in a biological medium using bioluminescence. More particularly, a method is provided for diagnosing diseases employing a solid phase 25 methodology and a luciferase-luciferin bioluminescence generating system. Methods employing biominerization for depositing silicon on a matrix support are also provided herein.

Luminescence, Narcosis and Life in the Deep Sea, Johnson, Vantage Press, NY, see, esp. pp. 50-56].

Though rare overall, bioluminescence is more common in marine organisms than in terrestrial organisms. Bioluminescence has developed 5 from as many as thirty evolutionarily distinct origins and, thus, is manifested in a variety of ways so that the biochemical and physiological mechanisms responsible for bioluminescence in different organisms are distinct. Bioluminescent species span many genera and include microscopic organisms, such as bacteria [primarily marine bacteria including *Vibrio* 10 species], fungi, algae and dinoflagellates, to marine organisms, including arthropods, mollusks, echinoderms, and chordates, and terrestrial organism including annelid worms and insects.

Bioluminescence, as well as other types of chemiluminescence, is used for quantitative determinations of specific substances in biology and 15 medicine. For example, luciferase genes have been cloned and exploited as reporter genes in numerous assays, for many purposes. Since the different luciferase systems have different specific requirements, they may be used to detect and quantify a variety of substances. The majority of commercial bioluminescence applications are based on firefly [*Photinus pyralis*] 20 luciferase. One of the first and still widely used assays involves the use of firefly luciferase to detect the presence of ATP. It is also used to detect and quantify other substrates or co-factors in the reaction. Any reaction that produces or utilizes NAD(H), NADP(H) or long chain aldehyde, either directly or indirectly, can be coupled to the light-emitting reaction of 25 bacterial luciferase.

Another luciferase system that has been used commercially for analytical purposes is the *Aequorin* system. The purified jellyfish photoprotein, aequorin, is used to detect and quantify intracellular Ca^{2+} and its changes under various experimental conditions. The *Aequorin*

may be practiced with any suitable chip device, including self-addressable and non-self addressable formats, that is modified as described herein for detection of generated photons by the bioluminescence generating systems.

The chip device provided herein is adaptable for use in an array format for

5 the detection and identification of infectious agents in biological specimens.

To prepare the chip, a suitable matrix for chip production is selected, the chip is fabricated by suitably derivatizing the matrix for linkage of macromolecules, and including linkage of photodiodes, photomultipliers CCD (charge coupled device) or other suitable detector, for measuring light

10 production; attaching an appropriate macromolecule, such as a biological molecule or anti-ligand, e.g., a receptor, such as an antibody, to the chip, preferably to an assigned location thereon. Photodiodes are presently among the preferred detectors, and specified herein. It is understood, however, that other suitable detectors may be substituted therefor.

15 In one embodiment, the chip is made using an integrated circuit with an array, such as an X-Y array, of photodetectors. The surface of circuit is treated to render it inert to conditions of the diagnostic assays for which the chip is intended, and is adapted, such as by derivatization for linking molecules, such as antibodies. A selected antibody or panel of antibodies, 20 such as an antibody specific for particularly bacterial antigen, is affixed to the surface of the chip above each photodetector. After contacting the chip with a test sample, the chip is contacted a second antibody linked to a component of a bioluminescence generating system, such as a luciferase or luciferin, specific for the antigen. The remaining components of the 25 bioluminescence generating reaction are added, and, if any of the antibodies linked to a component of a bioluminescence generating system are present on the chip, light will be generated and detected by the adjacent photodetector. The photodetector is operatively linked to a computer, which is programmed with information identifying the linked antibodies,

In another embodiment, a microelectronic device for detecting and identifying analytes in a fluid sample using light-emitting reactions is provided. The device includes a substrate, an array of micro-locations defined thereon for receiving the fluid sample to be analyzed, a separate

5 targeting agent attached to an attachment layer of each micro-location, and an independent photodetector optically coupled to each micro-location. Each targeting agent is, preferably, specific for binding a selected analyte that may be present in the received sample. Each photodetector generates a sensed signal responsive to photons of light emitted at the corresponding

10 micro-location when the selected analyte bound thereto is exposed to a secondary binding agent also specific for binding the selected analyte or the targeting agent-selected analyte complex and linked to one or more components of a light-emitting reaction. The chip is then reacted with the remaining components to emit the photons when the selected analyte is

15 present. An electronic circuit reads the sensed signal generated by each photodetector and generates output data signals therefrom that are indicative of the light emitted at each micro-location.

In yet another embodiment, a microelectronic device for detecting and identifying analytes in a biological sample using luciferase-luciferin bio-

20 luminescence is provided. The device includes a substrate, an array of micro-locations defined thereon for receiving the sample to be analyzed, a separate anti-ligand, such as a receptor antibody, attached to an attachment layer of each micro-location, and an independent photodetector optically coupled to each micro-location. Each receptor antibody is specific for

25 binding a selected analyte that may be present in the received sample. Each photodetector generates a sensed signal responsive to bioluminescence emitted at the corresponding micro-location when the selected analyte bound to the corresponding receptor antibody is exposed to a secondary antibody also specific to the selected analyte or to the receptor

signals therefrom indicative of the bioluminescence emitted at each micro-location by the reaction.

In a further embodiment, a system for detecting and identifying analytes in a biological sample using luciferase-luciferin bioluminescence is provided. The system includes: a microelectronic device including an array of micro-locations for receiving the sample; a separate receptor antibody attached to an attachment layer of each micro-location, each receptor antibody is specific for a selected analyte that may be present in the received sample; a photodetector that generates a sensed signal responsive to bioluminescence emitted at the corresponding micro-location when the selected analyte bound to the corresponding receptor antibody is exposed to a secondary antibody also specific to the selected analyte and linked to one of a luciferase and a luciferin, and is then reacted with the other of the luciferase and luciferin to generate the bioluminescence when the selected analyte is present, and an electronic circuit which reads the sensed signal from each photodetector and generates output data signals therefrom indicative of the bioluminescence emitted at each micro-location by the reaction. The system includes a processing instrument including an input interface circuit for receiving the output data signals indicative of the bioluminescence emitted at each micro-location, a memory circuit for storing a data acquisition array having a location associated with each micro-location, an output device for generating visible indicia in response to an output device signal and a processing circuit. The processing circuit reads the output data signals received by the input interface circuit, correlates these signals with the corresponding micro-locations, integrates the correlated output data signals for a desired time period by accumulating them in the data acquisition array, and generates the output device signal which, when applied to the output device, causes the output device to generate visible indicia related to the presence of the selected analytes.

or other suitable detector in the semiconductor layer and the signal is relayed to a processing unit, typically a computer. The processing unit displays the well or wells that are positive. Each well corresponds to a particular ligand, thereby permitting identification of the infectious agents.

5 All steps may be automated.

The design, fabrication, and uses of nonself-addressable and programmable, self-addressable and self-assembling microelectronic systems and devices which actively carry out controlled multi-step and multiplex reactions in microscopic formats for detecting the electromagnetic

10 emissions of a bioluminescent reaction are provided herein. The reactions include, but are not limited to, most molecular biological procedures, such as nucleic acid and protein nucleic acid hybridizations, antibody/antigen reactions, and related clinical diagnostics.

The resulting chips, which includes a silicon matrix and photodiodes
15 or other light detecting means, are provided. The silicon may be deposited using enzymatic deposition, similar to the enzymatic deposition by radiolarains and diatoms. Also provided are chips in which the absorption of silica or derivatives thereof is advantageously employed as a detection means. Such silica has an absorption maxima at about 705 nm, which is
20 the wavelength emitted by *Aristostomias* bioluminescence generating system. Enzymatic methods for depositing silicon on the surface of a matrix are also provided herein.

Also provided herein is a synthetic synapse. A suitable enzyme, particularly, acetylcholine esterase is fused to a luciferase, such as by
25 recombinant expression. The luciferase is either in an inactive or active conformation. Suitable mutations in either protein may be selected to insure that luciferase can undergo appropriate conformational changes as described herein. The resulting fusion is attached to a chip, such as a chip provided herein. Upon binding of the ligand to the enzyme, such as the

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FIGURE 6 is a system block diagram showing the microelectronics device of FIG. 1 mounted on an adaptor circuit board and serially interfaced to a computer programmed to read the serial output data stream, to correlate the output data with the array of micro-locations, to integrate the 5 data correlated with each micro-location for a predetermined time period set using an input device, to identify the analytes present in the biological medium by reference to an analyte map, and to display the results on an output device; and

FIGURE 7 shows the microelectronics device of FIG. 1 received on a 10 circuit board which does not require the user to directly handle the package.

FIGURE 8 is a schematic cross-sectional diagram of a three layer multi-well CCD chip (a chip containing a photodiode/CCD).

FIGURE 9 shows a blown-up schematic diagram of a multi-well CCD chip bottom layer and middle reflective layer and schematic diagram of an 15 individual well.

FIGURE 10 shows a blown-up schematic diagram of specific antibodies attached to the middle reflective layer of the multi-well CCD chip of Fig. 8.

FIGURE 11 is a cross-section of an individual well indicating the 20 relative positions of the CCD, reflective mirror layer and the cathode and anode. Antibodies attached to the middle reflective layer hang inverted above the photodiode. Bound antigen is detected using an antibody-luciferase fusion protein, and light generated from the bioluminescent reaction is detected by the photodiode and relayed to a processing unit for 25 identification.

FIGURE 12 is the cross-section of three self-addressable micro-locations fabricated using microlithographic techniques [see, International PCT application No. WO 96/01836]. Included are arrows denoting the positioning of photodiodes.

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2. Ctenophore and coelenterate systems
 - a. The aequorin system
 - (1) Aequorin photoprotein
 - (2) Luciferin
- 5 b. The *Renilla* system
3. Crustacean, particular *Cypridina* [*Vargula*], systems
 - a. *Vargula* luciferase
 - (1) Purification from *Cypridina*
 - (2) Preparation by Recombinant Methods
 - b. *Vargula* luciferin
 - c. Reaction
- 10 4. Insect bioluminescence generating systems including fireflies, click beetles, and other insect systems
 - a. Luciferase
 - b. Luciferin
 - c. Reaction
- 15 5. Bacterial systems
 - a. Luciferases
 - b. Luciferins
 - c. Reactions
- 20 6. Other systems
 - a. Dinoflagellate bioluminescence generating systems
 - b. Systems from molluscs, such as *Latia* and *Pholas*
 - c. Earthworms and other annelids
 - d. Glow worms
 - e. Marine polychaete worm systems
 - f. South American railway beetle
- 25

Thus, chemiluminescence involves the direct conversion of chemical energy to light energy. Bioluminescence refers to the subset of chemiluminescence reactions that involve luciferins and luciferases (or the photoproteins). Bioluminescence does not herein include phosphorescence.

5 As used herein, bioluminescence, which is a type of chemiluminescence, refers to the emission of light by biological molecules, particularly proteins. The essential condition for bioluminescence is molecular oxygen, either bound or free in the presence of an oxygenase, a luciferase, which acts on a substrate, a luciferin. Bioluminescence is

10 generated by an enzyme or other protein [luciferase] that is an oxygenase that acts on a substrate luciferin [a bioluminescence substrate] in the presence of molecular oxygen and transforms the substrate to an excited state, which upon return to a lower energy level releases the energy in the form of light.

15 As used herein, the substrates and enzymes for producing bioluminescence are generically referred to as luciferin and luciferase, respectively. When reference is made to a particular species thereof, for clarity, each generic term is used with the name of the organism from which it derives, for example, bacterial luciferin or firefly luciferase.

20 As used herein, luciferase refers to oxygenases that catalyze a light emitting reaction. For instance, bacterial luciferases catalyze the oxidation of flavin mononucleotide [FMN] and aliphatic aldehydes, which reaction produces light. Another class of luciferases, found among marine arthropods, catalyzes the oxidation of *Cypridina* [*Vargula*] luciferin, and

25 another class of luciferases catalyzes the oxidation of *Coleoptera* luciferin.

Thus, luciferase refers to an enzyme or photoprotein that catalyzes a bioluminescent reaction [a reaction that produces bioluminescence]. The luciferases, such as firefly and *Renilla* luciferases, that are enzymes which act catalytically and are unchanged during the bioluminescence generating

As used herein, bioluminescence substrate refers to the compound that is oxidized in the presence of a luciferase, and any necessary activators, and generates light. These substrates are referred to as luciferins, which are substrates that undergo oxidation in a bioluminescence reaction. These bioluminescence substrates include any luciferin or analog thereof or any synthetic compound with which a luciferase interacts to generate light. Preferred substrates are those that are oxidized in the presence of a luciferase or protein in a light-generating reaction. Bioluminescence substrates, thus, include those compounds that those of skill in the art recognize as luciferins. Luciferins, for example, include firefly luciferin, *Cypridina* [also known as *Vargula*] luciferin [coelenterazine], bacterial luciferin, as well as synthetic analogs of these substrates or other compounds that are oxidized in the presence of a luciferase in a reaction that produces bioluminescence.

As used herein, capable of conversion into a bioluminescence substrate means susceptible to chemical reaction, such as oxidation or reduction, that yields a bioluminescence substrate. For example, the luminescence producing reaction of bioluminescent bacteria involves the reduction of a flavin mononucleotide group (FMN) to reduced flavin mononucleotide (FMNH₂) by a flavin reductase enzyme. The reduced flavin mononucleotide [substrate] then reacts with oxygen [an activator] and bacterial luciferase to form an intermediate peroxy flavin that undergoes further reaction, in the presence of a long-chain aldehyde, to generate light. With respect to this reaction, the reduced flavin and the long chain aldehyde are substrates.

As used herein, bioluminescence system [or bioluminescence generating system] refers to the set of reagents required for a bioluminescence-producing reaction. Thus, the particular luciferase, luciferin and other substrates, solvents and other reagents that may be

methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical

5 properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers. In such instances, further purification might increase the

10 specific activity of the compound.

As used herein equivalent, when referring to two sequences of nucleic acids means that the two sequences in question encode the same sequence of amino acids or equivalent proteins. When "equivalent" is used in referring to two proteins or peptides, it means that the two proteins or

15 peptides have substantially the same amino acid sequence with only conservative amino acid substitutions [see, e.g., Table 2, below] that do not substantially alter the activity or function of the protein or peptide. When "equivalent" refers to a property, the property does not need to be present to the same extent [e.g., two peptides can exhibit different rates of the

20 same type of enzymatic activity], but the activities are preferably substantially the same. "Complementary," when referring to two nucleotide sequences, means that the two sequences of nucleotides are capable of hybridizing, preferably with less than 25%, more preferably with less than 15%, even more preferably with less than 5%, most preferably with no

25 mismatches between opposed nucleotides. Preferably the two molecules will hybridize under conditions of high stringency.

As used herein: stringency of hybridization in determining percentage mismatch is as follows:

- 1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C

proteins, organic molecules, nucleic acids, viruses, viral capsids, phage, cells or membranes thereof or portions of viruses, viral capsids, phage, cells or membranes. Of particular interest herein, are macromolecules that specifically bind to an analyte of interest. Analytes of interest are those

5 present in body fluids and other biological samples.

As used herein, a receptor refers to a molecule that has an affinity for a given ligand. Receptors may be naturally-occurring or synthetic molecules. Receptors may also be referred to in the art as anti-ligands. As used herein, the receptor and anti-ligand are interchangeable. Receptors

10 can be used in their unaltered state or as aggregates with other species.

Receptors may be attached, covalently or noncovalently, or in physical contact with, to a binding member, either directly or indirectly via a specific binding substance or linker. Examples of receptors, include, but are not limited to: antibodies, cell membrane receptors surface receptors and 15 internalizing receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants [such as on viruses, cells, or other materials], drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles.

Examples of receptors and applications using such receptors, include

20 but are not restricted to:

a) enzymes: specific transport proteins or enzymes essential to survival of microorganisms, which could serve as targets for antibiotic [ligand] selection;

b) antibodies: identification of a ligand-binding site on the antibody

25 molecule that combines with the epitope of an antigen of interest may be investigated; determination of a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related

As used herein, a ligand-receptor pair or complex formed when two macromolecules have combined through molecular recognition to form a complex.

As used herein, an epitope refers to a portion of an antigen molecule 5 that is delineated by the area of interaction with the subclass of receptors known as antibodies.

As used herein, a ligand is a molecule that is specifically recognized by a particular receptor. Examples of ligands, include, but are not limited to, agonists and antagonists for cell membrane receptors, toxins and 10 venoms, viral epitopes, hormones [e.g., steroids], hormone receptors, opiates, peptides, enzymes, enzyme substrates, cofactors, drugs, lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.

As used herein, an anti-ligand (AL.sub.i): An anti-ligand is a molecule 15 that has a known or unknown affinity for a given ligand and can be immobilized on a predefined region of the surface. Anti-ligands may be naturally-occurring or manmade molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Anti-ligands may be reversibly attached, covalently or noncovalently, to a binding member, 20 either directly or via a specific binding substance. By "reversibly attached" is meant that the binding of the anti-ligand (or specific binding member or ligand) is reversible and has, therefore, a substantially non-zero reverse, or unbinding, rate. Such reversible attachments can arise from noncovalent interactions, such as electrostatic forces, van der Waals forces, hydrophobic 25 (i.e., entropic) forces, and the like. Furthermore, reversible attachments also may arise from certain, but not all covalent bonding reactions. Examples include, but are not limited to, attachment by the formation of hemiacetals, hemiketals, imines, acetals, ketals, and the like (See, Morrison et al., "Organic Chemistry", 2nd ed., ch. 19 (1966), which is incorporated herein

and other materials used as supports for solid phase syntheses, affinity separations and purifications, hybridization reactions, immunoassays and other such applications.

As used herein, the attachment layer refers the surface of the chip device to which molecules are linked. Typically, the chip is a semiconductor device, which is coated on a least a portion of the surface to render it suitable for linking molecules and inert to any reactions to which the device is exposed. Molecules are linked either directly or indirectly to the surface, linkage may be effected by absorption or adsorption, through covalent bonds, ionic interactions or any other interaction. Where necessary the attachment layer is adapted, such as by derivatization for linking the molecules.

B. Bioluminescence generating systems

A bioluminescence generating system refers to the components that are necessary and sufficient to generate bioluminescence. These include a luciferase, luciferin and any necessary co-factors or conditions. Virtually any bioluminescence generating system known to those of skill in the art will be amenable to use in the apparatus, systems, combinations and methods provided herein. Factors for consideration in selecting a bioluminescence generating system, include, but are not limited to: the desired assay and biological fluid used in combination with the bioluminescence; the medium in which the reaction is run; stability of the components, such as temperature or pH sensitivity; shelf life of the components; sustainability of the light emission, whether constant or intermittent; availability of components; desired light intensity; and other such factors.

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	Type of Organism	Representative genera
	Annelids Earthworms Marine polychaetes Syllid fireworm	Diplocardia Chaetopterus, Phyxotrix Odontosyllis
5	Molluscs Limpet Clam Squid	Latia Pholas Heteroteuthis Heterocarpus
10	Crustacea Ostracod	Vargula (Cypridina)
15	Shrimp (euphausiids) Decapod Copepods	Meganyctiphanes Acanthophyra Oplophorus Gnathophausia Sergestes
20	Insects Coleopterids (beetles) Firefly Click beetles Railroad worm Diptera (flies)	Photinus, Photuris Pyrophorus Phengodes, Phrixothrix Arachnocampa
25	Echinoderms Brittle stars Sea cucumbers	Ophiopsila Laetmogone
	Chordates Tunicates	Pyrosoma

synthetic and alternative substrates have been devised. The DNA listed herein is only representative of the DNA encoding luciferases that is available.

Any bioluminescence generating system, whether synthetic or 5 isolated from natural sources, such as those set forth in Table 1, elsewhere herein or known to those of skill in the art, is intended for use in the chip devices, combinations, systems and methods provided herein. Chemiluminescence systems per se, which do not rely on oxygenases [luciferases] are not encompassed herein.

10 a. Luciferases

Luciferases refer to any compound that, in the presence of any necessary activators, catalyze the oxidation of a bioluminescence substrate [luciferin] in the presence of molecular oxygen, whether free or bound, from a lower energy state to a higher energy state such that the substrate, upon 15 return to the lower energy state, emits light. For purposes herein, luciferase is broadly used to encompass enzymes that act catalytically to generate light by oxidation of a substrate and also photoproteins, such as aequorin, that act, though not strictly catalytically [since such proteins are exhausted in the reaction], in conjunction with a substrate in the presence of oxygen 20 to generate light. These luciferases, including photoproteins, such as aequorin, are herein also included among the luciferases. These reagents include the naturally-occurring luciferases [including photoproteins], proteins produced by recombinant DNA, and mutated or modified variants thereof 25 that retain the ability to generate light in the presence of an appropriate substrate, co-factors and activators or any other such protein that acts as a catalyst to oxidize a substrate, whereby light is produced.

Generically, the protein that catalyzes or initiates the bioluminescent reaction is referred to as a luciferase, and the oxidizable substrate is referred to as a luciferin. The oxidized reaction product is termed oxyluciferin, and

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TABLE 2

	Original residue	Conservative substitution
	Ala (A)	Gly; Ser
5	Arg (R)	Lys
	Asn (N)	Gln; His
	Cys (C)	Ser; neutral amino acid
	Gln (Q)	Asn
	Glu (E)	Asp
10	Gly (G)	Ala; Pro
	His (H)	Asn; Gln
	Ile (I)	Leu; Val
	Leu (L)	Ile; Val
	Lys (K)	Arg; Gln; Glu
	Met (M)	Leu; Tyr; Ile
15	Phe (F)	Met; Leu; Tyr
	Ser (S)	Thr
	Thr (T)	Ser
	Trp (W)	Tyr
	Tyr (Y)	Trp; Phe
20	Val (V)	Ile; Leu

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions. Any such modification of the polypeptide may be effected by any means known to

25 those of skill in this art.

The luciferases may be obtained commercially, isolated from natural sources, expressed in host cells using DNA encoding the luciferase, or obtained in any manner known to those of skill in the art. For purposes herein, crude extracts obtained by grinding up selected source organisms

30 may suffice. Since large quantities of the luciferase may be desired, isolation of the luciferase from host cells is preferred. DNA for such purposes is widely available as are modified forms thereof.

Examples of luciferases include, but are not limited to, those isolated from the ctenophores *Mnemiopsis* (mnemiopsis) and *Beroe ovata* (berovin),

35 those isolated from the coelenterates *Aequorea* (aequorin), *Obelia* (obelin), *Pelagia*, the *Renilla* luciferase, the luciferases isolated from the mollusca *Pholas* (pholasin), the luciferases isolated from the *Aristostomias* and *Porichthys* fish and from the ostracods, such as *Cypridina* (also referred to

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Most of the systems provided herein will generate light when the luciferase and luciferin are mixed and exposed to air or water. The systems that use photoproteins that have bound oxygen, such as aequorin, however, will require exposure to Ca^{2+} [or other suitable metal ion], which can be

5 provided in the form of an aqueous composition of a calcium salt. In these instances, addition of a Ca^{2+} [or other suitable metal ion] to a mixture of luciferase [aequorin] and luciferin [such as coelenterazine] will result in generation of light. The *Renilla* system and other Anthozoa systems also require Ca^{2+} [or other suitable metal ion].

10 If crude preparations are used, such as ground up *Cypridina* [shrimp] or ground fireflies, it may be necessary to add only water. In instances in which fireflies [or a firefly or beetle luciferase] are used the reaction may only require addition ATP. The precise components will be apparent, in light of the disclosure herein, to those of skill in this art or may be readily 15 determined empirically.

It is also understood that these mixtures will also contain any additional salts or buffers or ions that are necessary for each reaction to proceed. Since these reactions are well-characterized, those of skill in the art will be able to determine precise proportions and requisite components.

20 Selection of components will depend upon the chip device and system, the assay to be preformed and the luciferase. Various embodiments are described and exemplified herein; in view of such description, other embodiments will be apparent.

d. Reactions

25 In all embodiments, up to all but one component of a bioluminescence generating system will be bound directly or indirectly to the appropriate locations of the chip or otherwise immobilized at those positions of the array in which the presence of analyte, preferably an infectious agent, is detected. When bioluminescence is desired, the remaining component(s)

per liter [or the amount of coating on substrate the results from contacting with such composition] of each component on the order of 0.1 to 20 mg, preferably 0.1 to 10 mg, more preferably between about 1 and 10 mg of each component will be sufficient. When preparing coated substrates, as

5 described herein, greater amounts of coating compositions containing higher concentrations of the luciferase or luciferin may be used.

Thus, for example, in presence of calcium, 5 mg of luciferin, such as coelenterazine, in one liter of water will glow brightly for at least about 10 to 20 minutes, depending on the temperature of the water, when about 10 10 mgs of luciferase, such as aequorin photoprotein luciferase or luciferase from *Renilla*, is added thereto. Increasing the concentration of luciferase, for example, to 100 mg/l, provides a particularly brilliant display of light.

If desired, the onset of the bioluminescent reaction can be delayed by adding an, an inhibitor, for example magnesium, of the bioluminescence 15 generating reaction. Also, where inhibition is not desired, the concentration of free magnesium may be reduced by addition of a sufficient amount of chelating agent, such as ethylenediaminetetraacetic acid [EDTA]. The amount of EDTA and also calcium can be empirically determined to appropriately chelate magnesium, without inhibiting or preventing the 20 desired bioluminescence.

It is understood, that concentrations and amounts to be used depend upon the selected luciferase, the desired bacterial target, the concentration and amount of light absorbed by the immobilized anti ligand, the size of the photodiode array and these may be readily determined empirically. 25 Proportions, particularly those used when commencing an empirical determination, are generally those used for analytical purposes, and amounts or concentrations are at least those used for analytical purposes, but the amounts can be increased, particularly if a sustained and brighter glow is desired.

Shimomura *et al.* (1981) "Resistivity to denaturation of the apoprotein of aequorin and reconstitution of the luminescent photoprotein from the partially denatured apoprotein," *Biochem. J.* **199**:825-828; Inouye *et al.* (1989) *J. Biochem.* **105**:473-477; Inouye *et al.* (1986) "Expression of 5 Apoaequorin Complementary DNA in *Escherichia coli*," *Biochemistry* **25**:8425-8429; Inouye *et al.* (1985) "Cloning and sequence analysis of cDNA for the luminescent protein aequorin," *Proc. Natl. Acad. Sci. USA* **82**:3154-3158; Prendergast, *et al.* (1978) "Chemical and Physical Properties of Aequorin and the Green Fluorescent Protein Isolated from *Aequorea forskalea*" *J. Am. Chem. Soc.* **17**:3448-3453; European Patent Application 0 540 064 A1; European Patent Application 0 226 979 A2, European Patent Application 0 245 093 A1 and European Patent Specification 0 245 093 B1; U.S. Patent No. 5,093,240; U.S. Patent No. 5,360,728; U.S. Patent No. 5,139,937; U.S. Patent No. 5,422,266; U.S. Patent No. 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 199 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 405 410 415 420 425 430 435 440 445 450 455 460 465 470 475 480 485 490 495 500 505 510 515 520 525 530 535 540 545 550 555 560 565 570 575 580 585 590 595 600 605 610 615 620 625 630 635 640 645 650 655 660 665 670 675 680 685 690 695 700 705 710 715 720 725 730 735 740 745 750 755 760 765 770 775 780 785 790 795 800 805 810 815 820 825 830 835 840 845 850 855 860 865 870 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Fed. Proc. 34:474]. DNA encoding numerous forms has been isolated [see, e.g., SEQ ID Nos. 5-9 and 13].

The photoprotein can be reconstituted [see, e.g., U.S. Patent No. 5,023,181] by combining the apoprotein, such as a protein recombinantly produced in E. coli, with a coelenterazine, such as a synthetic coelenterazine, in the presence of oxygen and a reducing agent [see, e.g., Shimomura et al. (1975) Nature 256:236-238; Shimomura et al. (1981) Biochemistry J. 199:825-828], such as 2-mercaptoethanol, and also EDTA or EGTA [concentrations between about 5 to about 100 mM or higher for applications herein] tie up any Ca^{2+} to prevent triggering the oxidation reaction until desired. DNA encoding a modified form of the apoprotein that does not require 2-mercaptoethanol for reconstitution is also available [see, e.g., U.S. Patent No. U.S. Patent No. 5,093,240]. The reconstituted photoprotein is also commercially available [sold, e.g., under the trademark AQUALITE[®], which is described in U.S. Patent No. 5,162,227].

The light reaction is triggered by adding Ca^{2+} at a concentration sufficient to overcome the effects of the chelator and achieve the 10^{-6} M concentration. Because such low concentrations of Ca^{2+} can trigger the reaction, for use in the methods herein, higher concentrations of chelator may be included in the compositions of photoprotein. Accordingly, higher concentrations of added Ca^{2+} in the form of a calcium salt will be required. Precise amounts may be empirically determined. For use herein, it may be sufficient to merely add water to the photoprotein, which is provided in the form of a concentrated composition or in lyophilized or powdered form. Thus, for purposes herein, addition of small quantities of Ca^{2+} , such as those present in most tap water or in phosphate buffered saline (PBS) or other suitable buffers or possible in the moisture on the skin, should trigger the bioluminescence reaction.

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Of interest herein, are forms of the apoprotein that have been modified so that the bioluminescent activity is greater than unmodified apoaequorin [see, e.g., U.S. Patent No. 5,360,728, SEQ ID Nos. 10-12]. Modified forms that exhibit greater bioluminescent activity than unmodified

5 apoaequorin include proteins having sequences set forth in SEQ ID Nos. 10-12, in which aspartate 124 is changed to serine, glutamate 135 is changed to serine, and glycine 129 is changed to alanine, respectively. Other modified forms with increased bioluminescence are also available.

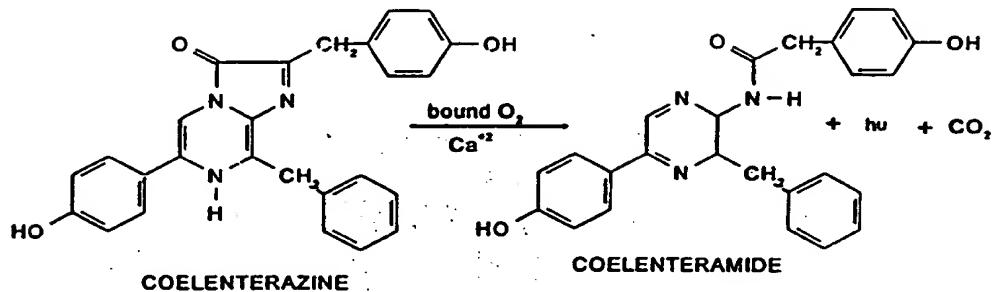
For use in certain embodiments herein, the apoprotein and other 10 components of the aequorin bioluminescence generating system are packaged or provided as a mixture, which, when desired is subjected to conditions under which the photoprotein reconstitutes from the apoprotein, luciferin and oxygen [see, e.g., U.S. Patent No. 5,023,181; and U.S. Patent No. 5,093,240]. Particularly preferred are forms of the apoprotein that do 15 not require a reducing agent, such as 2-mercaptoethanol, for reconstitution. These forms, described, for example in U.S. Patent No. 5,093,240 [see, also Tsuji et al. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:8107-8111], are modified by replacement of one or more, preferably all three cysteine residues with, for example serine. Replacement may be effected by 20 modification of the DNA encoding the aequorin apoprotein, such as that set forth in SEQ ID No. 5, and replacing the cysteine codons with serine.

The photoproteins and luciferases from related species, such as *Obelia* are also contemplated for use herein. DNA encoding the Ca^{2+} -activated photoprotein obelin from the hydroid polyp *Obelia longissima* is known 25 and available [see, e.g., Illarionov et al. (1995) Gene 153:273-274; and Bondar et al. (1995) Biochim. Biophys. Acta 1231:29-32]. This photoprotein can also be activated by Mn^{2+} [see, e.g., Vysotski et al. (1995) Arch. Bioch. Biophys. 316:92-93, Vysotski et al. (1993) J. Biolumin. Chemilumin. 8:301-305].

and sulfated derivatives thereof.

The reaction of coelenterazine when bound to the aequorin

5 photoprotein with bound oxygen and in the presence of Ca^{2+} can represented as follows:



The photoprotein aequorin [which contains apoaequorin bound to a coelenterate luciferin molecule] and *Renilla* luciferase, discussed below, can use the same coelenterate luciferin. The aequorin photoprotein catalyses

10 the oxidation of coelenterate luciferin [coelenterazine] to oxyluciferin [coelenteramide] with the concomitant production of blue light [$\lambda_{\text{max}} = 469 \text{ nm}$].

Importantly, the sulfate derivative of the coelenterate luciferin [lauryl-luciferin] is particularly stable in water, and thus may be used in a
 15 coelenterate-like bioluminescence generating system. In this system, adenosine diphosphate (ADP) and a sulpha-kinase are used to convert the coelenterazine to the sulphated form. Sulfatase is then used to reconvert the lauryl-luciferin to the native coelenterazine. Thus, the more stable lauryl-luciferin is used in the item to be illuminated and the luciferase
 20 combined with the sulfatase are added to the luciferin mixture when illumination is desired.

Thus, the bioluminescence generating system of *Aequorea* is particularly suitable for use in the methods and apparatus herein. The

Ophophorus, *Sergestes*, and *Gnathophausia*; deep-sea fish, such as *Argyropelecus*, *Yarella*, *Diaphus*, and *Neoscopelus*.

Renilla luciferase does not, however, have bound oxygen, and thus requires dissolved oxygen in order to produce light in the presence of a suitable luciferin substrate. Since *Renilla* luciferase acts as a true enzyme [i.e., it does not have to be reconstituted for further use] the resulting luminescence can be long-lasting in the presence of saturating levels of luciferin. Also, *Renilla* luciferase is relatively stable to heat.

Renilla luciferase, DNA encoding *Renilla* luciferase, and use of the DNA to produce recombinant luciferase, as well as DNA encoding luciferase from other coelenterates, are well known and available [see, e.g., SEQ ID No. 1, U.S. Patent Nos. 5,418,155 and 5,292,658; see, also, Prasher *et al.* (1985) *Biochem. Biophys. Res. Commun.* **126**:1259-1268; Cormier (1981) "Renilla and Aequorea bioluminescence" in *Bioluminescence and Chemiluminescence*, pp. 225-233; Charbonneau *et al.* (1979) *J. Biol. Chem.* **254**:769-780; Ward *et al.* (1979) *J. Biol. Chem.* **254**:781-788; Lorenz *et al.* (1981) *Proc. Natl. Acad. Sci. U.S.A.* **88**: 4438-4442; Hori *et al.* (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**:4285-4287; Hori *et al.* (1975) *Biochemistry* **14**:2371-2376; Hori *et al.* (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**:4285-4287; Inouye *et al.* (1975) *Jap. Soc. Chem. Lett.* **141**-144; and Matthews *et al.* (1979) *Biochemistry* **16**:85-91]. The DNA encoding *Renilla* luciferase and host cells containing such DNA provide a convenient means for producing large quantities of the enzyme [see, e.g., U.S. Patent Nos. 5,418,155 and 5,292,658, which describe recombinant production of *Renilla* luciferase and the use of the DNA to isolate DNA encoding other luciferases, particularly those from related organisms]. A modified version of a method [U.S. Patent Nos. 5,418,155 and 5,292,658] for the recombinant production of *Renilla* luciferase that results in a higher level of

luciferase may also be linked to an anti ligand through chemical or recombinant means for use in the methods herein.

Recombinant production of *Renilla reniformis* luciferase

The phagemid pTZ18R (Pharmacia) is a multi-purpose DNA vector

5 designed for in vitro transcriptions and useful for expression of recombinant proteins in bacterial hosts. The vector contains the bla gene, which allows for the selection of transformants by resistance to ampicillin, and a polylinker site adjacent to the lacZ' gene. The heterologous gene of interest is inserted in the polylinker and transcribed
10 from the lac promoter by induction, for example, with isopropyl- β -D-thiogalactopyranoside (IPTG).

The DNA encoding the *Renilla reniformis* luciferase has been cloned (e.g., see U.S. Patent Nos. 5,292,658 and 5,418,155). The plasmid pTZRLuc-1 encodes the *Renilla* luciferase on a 2.2 Kbp EcoRI to
15 SstI DNA fragment inserted in EcoRI and SstI sites of pTZ18R (plasmid construction is described U.S. Patent Nos. 5,292,658 and 5,418,155; see also Lorenz et al. (1991) Isolation and Expression of a cDNA encoding *Renilla reniformis* Luciferase, Proc. Natl. Acad. Sci. U.S.A. 88, 4438-4442). The initiation of transcription of the *Renilla* luciferase cDNA
20 is under the control of the lacZ' promoter. E. coli strains harboring plasmid pTZRLuc-1 express *Renilla* luciferase that is functional in bioluminescence assays and retains the properties of the native enzyme (see e.g., U.S. Patent Nos. 5,292,658 and 5,418,155).

A derivative of pTZRLuc-1, pTZRLuc-3.6, produces approximately
25 7-fold higher levels of recombinant *Renilla* luciferase than pTZRLuc-1 when transformed into the same E. coli host. Competent E. coli strain XL-1 was transformed using purified pTZRLuc-3.6 according to the instructions provided by the manufacturer (XL-1 Supercompetent cells and protocol; Stratagene, Inc., La Jolla, CA). Transfectants were

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3. Crustacean, particularly *Cypridina* systems

The ostracods, such as *Vargula serrata*, *hilgendorfii* and *noctiluca* are small marine crustaceans, sometimes called sea fireflies. These sea fireflies are found in the waters off the coast of Japan and emit light by

5 squirting luciferin and luciferase into the water, where the reaction, which produces a bright blue luminous cloud, occurs. The reaction involves only luciferin, luciferase and molecular oxygen, and, thus, is very suitable for application herein.

The systems, such as the *Vargula* bioluminescence generating

10 systems, are particularly preferred herein because the components are stable at room temperature if dried and powdered and will continue to react even if contaminated. Further, the bioluminescent reaction requires only the luciferin/luciferase components in concentrations as low as 1:40 parts per billion to 1:100 parts per billion, water and molecular oxygen to 15 proceed. An exhausted system can be renewed by addition of luciferin.

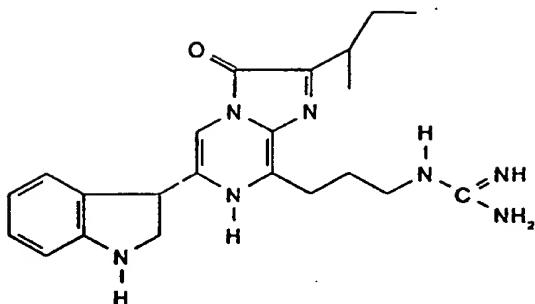
a. *Vargula* luciferase

Vargula luciferase is a 555-amino acid polypeptide that has been produced by isolation from *Vargula* and also using recombinant technology by expressing the DNA in suitable bacterial and mammalian 20 host cells [see, e.g., Thompson *et al.* (1989) Proc. Natl. Acad. Sci. U.S.A. 86:6567-6571; Inouye *et al.* (1992) Proc. Natl. Acad. Sci. U.S.A. 89:9584-9587; Johnson *et al.* (1978) Methods in Enzymology LVII:331-349; Tsuji *et al.* (1978) Methods Enzymol. 57:364-72; Tsuji (19740 Biochemistry 13:5204-5209; Japanese Patent Application No. JP 3-25 30678 Osaka; and European Patent Application No. EP 0 387 355 A1].

(1) Purification from *Cypridina*

Methods for purification of *Vargula* [*Cypridina*] luciferase are well known. For example, crude extracts containing the active can be readily prepared by grinding up or crushing the *Vargula* shrimp. In other

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Analogs thereof and other compounds that react with the luciferase in a light producing reaction also may be used.

15 Other bioluminescent organisms that have luciferases that can react with the *Vargula* luciferin include, the genera *Apogon*, *Parapriacanthus* and *Porichthys*.

c. Reaction

The luciferin upon reaction with oxygen forms a dioxetanone

20 intermediate [which includes a cyclic peroxide similar to the firefly cyclic peroxide molecule intermediate]. In the final step of the bioluminescent reaction, the peroxide breaks down to form CO₂ and an excited carbonyl. The excited molecule then emits a blue to blue-green light.

The optimum pH for the reaction is about 7. For purposes herein, 25 any pH at which the reaction occurs may be used. The concentrations of reagents are those normally used for analytical reactions or higher [see, e.g., Thompson *et al.* (1990) *Gene* 96:257-262]. Typically concentrations of the luciferase between 0.1 and 10 mg/l, preferably 0.5 to 2.5 mg/l will be used. Similar concentrations or higher concentrations 30 of the luciferin may be used.

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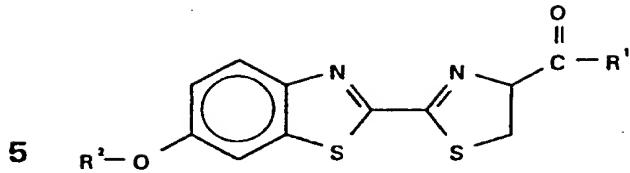
homology with luciferases from *Photinus pyralis*, *Luciola mingrellica*, *L. cruciata* or *L. lateralis* and having luciferase activity is available [see, e.g., International PCT Application No. WO95/25798]. It is more stable above 30° C than naturally-occurring insect luciferases and may also be

5 produced at 37° C or above, with higher yield.

Modified luciferases that generate light at different wavelengths [compared with native luciferase], and thus, may be selected for their color-producing characteristics. For example, synthetic mutant beetle luciferase(s) and DNA encoding such luciferases that produce

10 bioluminescence at a wavelength different from wild-type luciferase are known [Promega Corp, International PCT Application No. WO95/18853, which is based on U.S. application Serial No. 08/177,081 1/3/94]. The mutant beetle luciferase has an amino acid sequence differing from that of the corresponding wild-type *Luciola cruciata* [see, e.g., U.S. Patent
15 Nos. 5,182,202, 5,219,737, 5,352,598, see, also SEQ ID No.3] by a substitution(s) at one or two positions. The mutant luciferase produces a bioluminescence with a wavelength of peak intensity that differs by at least 1 nm from that produced by wild-type luciferases.

Other mutant luciferase have also been produced. Mutant
20 luciferases with the amino acid sequence of wild-type luciferase, but with at least one mutation in which valine is replaced by isoleucine at the amino acid number 233, valine by isoleucine at 239, serine by asparagine at 286, glycine by serine at 326, histidine by tyrosine at 433 or proline by serine at 452 are known [see, e.g., U.S. Patent Nos. 5,219,737, and
25 5,330,906]. The luciferases are produced by expressing DNA-encoding each mutant luciferase in *E. coli* and isolating the protein. These luciferases produce light with colors that differ from wild-type. The mutant luciferases catalyze luciferin to produce red [λ 609 nm and 612 nm], orange [λ 595 and 607 nm] or green [λ 558 nm] light. The other



in which:

R¹ is hydroxy, amino,

linear or branched C₁-C₂₀ alkoxy,

C₂-C₂₀ alkylenoxy, an L-amino acid radical bond via the α -amino group, an oligopeptide radical with up to ten L-amino acid units linked via the α -amino group of the terminal unit;

10 R² is hydrogen, H₂PO₃, HSO₃, unsubstituted or phenyl substituted linear or branched C₁-C₂₀ alkyl or C₂-C₂₀ alkenyl, aryl containing 6 to 18 carbon atoms, or R³-C(O)-; and

R³ is an unsubstituted or phenyl substituted linear or branched C₁-C₂₀ alkyl or C₂-C₂₀ alkenyl, aryl containing 6 to 18 carbon atoms, a

15 nucleotide radical with 1 to 3 phosphate groups, or a glycosidically attached mono- or disaccharide, except when formula (IV) is a D-luciferin or D-luciferin methyl ester.

c. Reaction

The reaction catalyzed by firefly luciferases and related insect

20 luciferases requires ATP, Mg²⁺ as well as molecular oxygen. Luciferin must be added exogenously. Firefly luciferase catalyzes the firefly luciferin activation and the subsequent steps leading to the excited product. The luciferin reacts with ATP to form a luciferyl adenylate intermediate. This intermediate then reacts with oxygen to form a cyclic

25 luciferyl peroxy species, similar to that of the coelenterate intermediate cyclic peroxide, which breaks down to yield CO₂ and an excited state of the carbonyl product. The excited molecule then emits a yellow light; the color, however, is a function of pH. As the pH is lowered the color of the bioluminescence changes from yellow-green to red.

[see, e.g., Cohn *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* **90**:102-123].

These subunits associate to form a 2-chain complex luciferase enzyme, which catalyzes the light emitting reaction of bioluminescent bacteria, such as *Vibrio harveyi* [U.S. Patent No. 4,581,335; Belas *et al.* (1982)

5 *Science* **218**:791-793], *Vibrio fischeri* [Engebrecht *et al.* (1983) *Cell* **32**:773-781; Engebrecht *et al.* (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**:4154-4158] and other marine bacteria.

Bacterial luciferase genes have been cloned [see, e.g., U.S. Patent No. 5,221,623; U.S. Patent No. 4,581,335; European Patent Application

10 No. EP 386 691 A]. Plasmids for expression of bacterial luciferase, such as *Vibrio harveyi*, include pFIT001 (NRRL B-18080), pPALE001 (NRRL B-18082) and pMR19 (NRRL B-18081)] are known. For example the sequence of the entire *lux* regulon from *Vibrio fischeri* has been determined [Baldwin *et al.* (1984), *Biochemistry* **23**:3663-3667; Baldwin 15 *et al.* (1981) *Biochem.* **20**: 512-517; Baldwin *et al.* (1984) *Biochem.* **23**:3663-3667; see, also, e.g., U.S. Patent Nos. 5,196,318, 5,221,623, and 4,581,335]. This regulon includes *luxI* gene, which encodes a protein required for autoinducer synthesis [see, e.g., Engebrecht *et al.* (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**:4154-4158], the *luxC*, *luxD*, and 20 *luxE* genes, which encode enzymes that provide the luciferase with an aldehyde substrate, and the *luxA* and *luxB* genes, which encode the alpha and beta subunits of the luciferase.

Lux genes from other bacteria have also been cloned and are available [see, e.g., Cohn *et al.* (1985) *J. Biol. Chem.* **260**:6139-6146;

25 U.S. Patent No. 5,196,524, which provides a fusion of the *luxA* and *luxB* genes from *Vibrio harveyi*. Thus, luciferase alpha and beta subunit-encoding DNA is provided and can be used to produce the luciferase. DNA encoding the α [1065 bp] and β [984 bp] subunits, DNA encoding a luciferase gene of 2124 bp, encoding the alpha and beta subunits, a

frozen into ice or placed in solutions stored below 0 °C. After incubation at temperatures near 0 °C, the chip can be transferred to warmer temperatures and the reaction will proceed thereby providing a sustained glow.

5 Bacterial luciferase catalyzes the flavin-mediated hydroxylation of a long-chain aldehyde to yield carboxylic acid and an excited flavin; the flavin decays to ground state with the concomitant emission of blue green light [$\lambda_{max} = 490$ nm; see, e.g., Legocki et al. (1986) Proc. Natl. Acad. Sci. USA 81:9080; see U.S. Patent No. 5,196,524]:



10 The reaction can be initiated by contacting reduced flavin mononucleotide [FMNH₂] with a mixture of the bacterial luciferase, oxygen, and a long-chain aldehyde, usually n-decyl aldehyde.

DNA encoding luciferase from the fluorescent bacterium

15 *Alteromonas hanedai* is known [CHISSO CORP; see, also, Japanese application JP 7222590, published August 22, 1995]. The reduced flavin mononucleotide [FMNH₂; luciferin] reacts with oxygen in the presence of bacterial luciferase to produce an intermediate peroxy flavin. This intermediate reacts with a long-chain aldehyde [tetradecanal] to

20 form the acid and the luciferase-bound hydroxy flavin in its excited state. The excited luciferase-bound hydroxy flavin then emits light and dissociates from the luciferase as the oxidized flavin mononucleotide [FMN] and water. In vivo FMN is reduced again and recycled, and the aldehyde is regenerated from the acid.

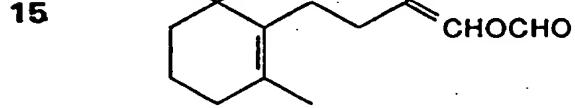
25 Flavin reductases have been cloned [see, e.g., U.S. Patent No. 5,484,723; see, SEQ ID No. 14 for a representative sequence from this patent]. These as well as NAD(P)H can be included in the reaction to regenerate FMNH₂ for reaction with the bacterial luciferase and long

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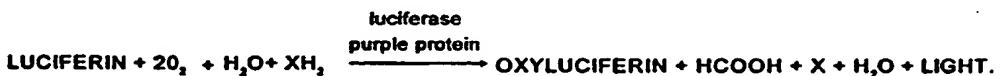
and Chemiluminescence, DeLuca *et al.*, eds. Academic Press, NY, pp.343-360]. Luminescent activity can be obtained in extracts made at pH 8 by shifting the pH from 8 to 6. This occurs in soluble and particulate fractions. Within the intact scintillon, the luminescent flash 5 occurs for ~100 msec, which is the duration of the flash *in vivo*. In solution, the kinetics are dependent on dilution, as in any enzymatic reaction. At pH 8, the luciferin is bound to a protein [luciferin binding protein] that prevents reaction of the luciferin with the luciferase. At pH 6, however, the luciferin is released and free to react with the enzyme.

10 b. Systems from molluscs, such as *Latia* and *Pholas*

Molluscs *Latia neritoides* and species of *Pholas* are bioluminescent animals. The luciferin has the structure:



20 and has been synthesized [see, *e.g.*, Shimomura *et al.* (1968) *Biochemistry* **7**:1734-1738; Shimomura *et al.* (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**:2086-2089]. In addition to a luciferase and luciferin the reaction has a third component, a "purple protein". The reaction, which can be initiated by an exogenous reducing agent is represented by the 25 following scheme:



XH_2 is a reducing agent.

Thus for practice herein, the reaction will require the purple protein as well as a reducing agent.

7. Fluorescent Proteins

a. Green and blue fluorescent proteins

As described herein, blue light is produced using the *Renilla* luciferase or the *Aequorea* photoprotein in the presence of Ca^{2+} and the 5 coelenterazine luciferin or analog thereof. This light can be converted into a green light if a green fluorescent protein (GFP) is added to the reaction. Green fluorescent proteins, which have been purified [see, e.g., Prasher *et al.* (1992) *Gene* 111:229-233] and also cloned [see, e.g., International PCT Application No. WO 95/07463, which is based on U.S. 10 application Serial No. 08/119,678 and U.S. application Serial No. 08/192,274, which are herein incorporated by reference], are used by cnidarians as energy-transfer acceptors. GFPs fluoresce *in vivo* upon receiving energy from a luciferase-oxyluciferein excited-state complex or a Ca^{2+} -activated photoprotein. The chromophore is modified amino acid 15 residues within the polypeptide. The best characterized GFPs are those of *Aequorea* and *Renilla* [see, e.g., Prasher *et al.* (1992) *Gene* 111:229-233; Hart, *et al.* (1979) *Biochemistry* 18:2204-2210]. For example, a 20 green fluorescent protein [GFP] from *Aequorea victoria* contains 238 amino acids, absorbs blue light and emits green light. Thus, inclusion of this protein in a composition containing the aequorin photoprotein 25 charged with coelenterazine and oxygen, can, in the presence of calcium, result in the production of green light. Thus, it is contemplated that GFPs may be included in the bioluminescence generating reactions that employ the aequorin or *Renilla* luciferases or other suitable luciferase in order to enhance or alter color of the resulting bioluminescence.

GFPs are activated by blue light to emit green light and thus may be used in the absence of luciferase and in conjunction with an external light source with novelty items, as described herein. Similarly, blue fluorescent proteins (BFPs), such as from *Vibrio fischeri*, *Vibrio harveyi* or

proteins have been used as fluorescent labels in immunoassay [see, Kronick (1986) J. of Immunolog. Meth. **92**:1-13], the proteins have been isolated and DNA encoding them is also available [see, e.g., Pilot et al. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**:6983-6987; Lui et al. (1993)

5 Plant Physiol **103**:293-294; and Houmard et al. (1988) J. Bacteriol. **170**:5512-5521; the proteins are commercially available from, for example, ProZyme, Inc., San Leandro, CA].

In these organisms, the phycobiliproteins are arranged in subcellular structures termed phycobilisomes and function as accessory 10 pigments that participate in photosynthetic reactions by absorbing visible light and transferring the derived energy to chlorophyll via a direct fluorescence energy transfer mechanism.

Two classes of phycobiliproteins are known based on their color: phycoerythrins (red) and phycocyanins (blue), which have reported 15 absorption maxima between 490 and 570 nm and between 610 and 665 nm, respectively. Phycoerythrins and phycocyanins are heterogenous complexes composed of different ratios of alpha and beta monomers to which one or more class of linear tetrapyrrole chromophores are covalently bound. Particular phycobiliproteins may 20 also contain a third γ -subunit which often associates with $(\alpha\beta)_6$ aggregate proteins.

All phycobiliproteins contain phycotrehobilin or phycoerythobilin chromophores, and may also contain other bilins, such as phycourobilin, cryptoviolin or a 697 nm bilin. The γ -subunit is covalently bound with 25 phycourobilin, which results in the 495-500 nm absorbance peak of B- and R-phycoerythrins. Thus, the spectral characteristics of phycobiliproteins may be influenced by the combination of the different chromophores, the subunit composition of the apo-phycobiliproteins

1. Nonself-addressable chips

Referring to FIG. 1, a nonself-addressable microelectronic device 100 for detecting and identifying analytes in a biological sample using bioluminescence includes an address control circuit 102, a photodetector array 104, an analog multiplexer 106, a comparator 108, a reference circuit 110, a feedback control circuit 112 and an output control circuit 114. Address control circuit 102 receives a clock input signal 116 from an external oscillator, and output control circuit 114 generates data output signals 118. Device 100 also includes electrical connections 120 and 122 for receiving electrical power and ground, respectively, from an external power source (e.g., an AC-DC converter). Thus, device 100 requires only four electrical connections: clock input signal 116; data output signals 118; power 120; and ground 122.

Address control circuit 102 receives clock input signal 116 and generates address signals on busses 124-128 in response thereto which sequentially address each pixel element within array 104. Each pixel element has a row and a column address that are used to address the pixel. Address control circuit 102 sequentially addresses each row of pixel elements within array 104 using row address signals asserted on bus 124. For each row, address control circuit 102 generates address signals on bus 126 that are used as select signals by analog multiplexer 106, and also generates address signals on bus 128 that are used by feedback control circuit 112 to generate feedback signals for the pixel elements as described below. Address control circuit 102 generates binary address signals decoded into individual row and column address enable signals by one or more address decode circuits located in address control circuit 102, array 104, multiplexer 106 and/or feedback control circuit 112. The addressing of an array in electronic circuits is well known to those of ordinary skill in the art.

for each pixel element in array 104, and the sync signal is an arbitrary value (e.g., a byte having a value of decimal 255) used as a control signal to identify the start of each data frame. The external computer waits for the sync signal before correlating the received frame data to the 5 appropriate pixel elements in array 104. Alternatively, output control circuit 114 could include labels in the serial data stream that identify the pixel elements. A parallel data interface can also be used.

As will become apparent from the description below, array 104 includes pixel elements located at an array of micro-locations on the 10 surface of the semiconductor substrate used for device 100. Each element includes a photodetector for receiving photons of light emitted by a chemical reaction optically coupled at the respective micro-location and for converting the received photons into an electric charge. Each element also includes a pixel unit cell circuit with a capacitance circuit for 15 integrating the electric charge. The integrated charge is quantized using delta-sigma A/D conversion techniques, and the digitized signal is multiplexed into a serial data output stream interfaced to an external computer. The computer executes a control program to integrate the delta-sigma digital signal for a desired integration period ranging from 20 seconds to hours depending on the desired resolution. In one embodiment, the delta-sigma A/D conversion is clocked for a 56 Kbaud interface to achieve 12-bit resolution in an integration period of about 10 seconds, and 16-bit resolution in a time period of about 3 minutes.

Referring to FIG. 2, device 100 includes a semiconductor substrate 25 or die 140 having array 104 defined on a surface thereof. Array 104 includes an array of micro-locations 142, and an independent photodetector 144 optically coupled to each micro-location. (Only the left-most micro-location 142 and photodetector 144 in each row are labeled in FIG. 2 for clarity.) Array 104 includes three sub-arrays 146,

smaller pixel elements (e.g., 50 um) since a greater number of receptor antibodies can be bound to their larger surface areas, as explained herein, such that more photons of light will be emitted when a chemical reaction occurs at the respective micro-location. The smaller pixels can

5 be used to form a larger matrix on a given die size to allow a greater number of assays to be performed simultaneously. The optimum pixel size for detecting a particular analyte may be determined empirically.

The use of different-sized pixel elements on device 100 has two advantages. First, larger pixel elements can be used to detect analytes

10 requiring larger sensitivities while smaller pixel elements can be used to increase the number of pixel elements in the matrix for analytes having lower sensitivities. Second, different sizes can be used to help determine the optimum size for a particular analyte by empirical testing, with the optimum size being used for other embodiments of array 104.

15 Alternative arrangements of array 104 will be apparent to a person of ordinary skill in the art. For example, array 104 can include sub-arrays of pixel elements having different sizes (as in FIG. 2), or an array having only a single pixel size (e.g., a 12x16 array of 50 micron pixels). The size of each pixel (e.g., 50, 100, 200 microns in FIG. 2) can be modified

20 (e.g., a 400 micron pixel can be used). Also, the number of pixels in the array or sub-array (e.g., 4x16, 2x8 or 1x16 in FIG. 2) can be changed to include an $n \times m$ array or sub-array having n rows and m columns, n and m being integers. Further, shapes other than squares can be used for each pixel element (e.g., rectangles or circles). The size of die 140 can

25 be modified to accommodate the different arrangements of array 104, although use of a larger die may increase the cost of device 100. Also, die 140 may include more or fewer bonding pads 152, provided there are separate pads for clock input signal 116, data output signals 118, electrical power 120 and ground 122 (FIG. 1).

Bond wires 188 are coated with a material (e.g., epoxy) impervious to the fluid sample to be analyzed. The other conductive components of package 154, except for pins 156, may also be coated with the material to prevent direct contact with the fluid sample. Pins 5 156 of package 154 are not coated by the material such that pins 156 will make electrical contact with an external computer or instrument when package 154 is read thereby.

When performing an assay, the fluid sample to be analyzed is applied through apertures 172 and 182 to the surface of die 140 (and 10 micro-locations 142 formed thereon) housed within package 154. The fluid sample may be applied by pipetting the fluid sample into the test well formed by apertures 172 and 182, or simply by dipping package 154 into a container (now shown) filled with the sample. The electrical components of device 100 are protected from the sample by the 15 materials of package 154 itself, or by the epoxy coating. After the fluid sample is applied, the remaining components needed to cause light-emitting reactions optically coupled to micro-locations 142 are also applied to the surface of die 140 through apertures 172 and 182. The resulting light-emitting reactions are then detected by photodetectors 20 144 as described below in relation to FIG. 4.

Referring to FIG. 4, the photodetector 144 of each pixel element in array 104 includes a pixel unit cell circuit 200 associated therewith. Each photodetector 144 is preferably a photodiode that generates sensed signals (i.e., photocurrents) in response to photons of light 202 impinging 25 on its surface. Each pixel unit cell circuit 200 integrates this sensed signal and quantizes the integrated signal using delta-sigma A/D conversion techniques. Circuit 200 includes five MOSFET transistors T₁-T₅ designated by numerals 204-212, each having a gate terminal G, a source terminal S (with an arrow pointing in toward the oxide layer), a

light is present, Node 1 is still discharged due to the leakage component of I_D . The photocurrent component of I_D can be separated from the leakage current component by taking dark readings when the light-emitting reactions are not occurring, taking test readings when the 5 reactions are taking place, and correcting the test readings using the dark readings (e.g., by subtracting the dark readings from the test readings). The dark readings may be taken either before, after, or both before and after, the actual test takes place.

Referring back to FIG. 1 for a moment, the output currents flowing 10 from output terminal 220, designated I_{OUT} , are the column output signals 132 that are multiplexed by analog multiplexer 106 to form multiplexed output signals 134 input to comparator 108. Comparator 108 maintains I_{OUT} at a constant voltage since the sensed signal is a current, and generates quantized output signals 138 based upon comparisons 15 between signals 134 and reference current 136. Feedback control circuit 112 generates feedback signals 130, that form enable signals F_{en} (216), based upon quantized output signals 138 and address signals 128. F_{en} for each pixel element is generated during the time period when the next pixel element is being addressed. When address control circuit 102 20 addresses the next row of pixel elements, causing R_{en} (214) to be asserted for that row, the next row enabled signal R_{en1} (218) is also asserted for the previous row of pixel elements using address decode circuits as are well known in the art.

Returning to FIG. 4, pixel unit cell circuit 200 operates as follows. 25 Photons 202 impinging on photodiode 144 generate current I_D that discharges Node 1 at a rate depending on the number of photons of light received, the photodiode's quantum efficiency, and the constant leakage current. When this pixel element is addressed by address control circuit 102 (i.e., R_{en} activated), transistors T_1 and T_5 are enabled (i.e., become

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After being reset to its initial value, the capacitance at Node 1 is again discharged by photodiode 144 at a rate dependent on the magnitude of I_D until I_{OUT} again transitions above reference current 136, at which time Node 1 is again recharged. Thus, Node 1 is kept at a

5 voltage near the voltage value required for T_4 to produce reference current 136. The number of times that comparator 108 senses reference current 136 exceeded (i.e., "comparator positive transitions") is proportional to the total charge that has flowed through photodiode 144. As stated previously, I_D is the sum of the constant leakage current and

10 the current due to sensed photons 202. Thus, the number of comparator positive transitions over a period of time can be used to acc-emitting reaction, after the number is adjusted for the leakage current flowing through photodiode 144 by subtracting the dark readings. Quantized output signals 138, as stated above, are formatted into an RS-232 serial

15 data stream transmitted to an external computer as data output signals 118.

Referring to FIG. 5, the voltages at Nodes 1, 2 and 3 during operation of device 100 are shown. The voltages at Nodes 1, 2 and 3 are designated by curves 222, 224 and 226, respectively. The x-axis represents time (msec), and the y-axis represents voltage (V). Voltages at nodes 1 and 3 are essentially equal during most of the downward sloping portions of curves 222 and 226, differing as shown in FIG. 5. At the start of each cycle (i.e., at each comparator positive transition occurring at each large spike in voltage at Node 3), the voltage at Node 1

20 is recharged to its initial value when Node 3 is set to V_{DD} . Then, Node 1 is discharged by I_D at a rate depending on the amount of light detected by photodiode 144. A steep decreasing slope on curve 222 occurs when photodiode 144 receives a relatively large amount of light, while a gradually decreasing slope occurs when photodiode 144 receives

Computer 304 includes a processing circuit 318, a memory circuit 320, and a serial interface circuit 322. Processing circuit 318 includes a central processing unit such as a microprocessor or microcontroller that receives input signals 324 from input device 306 and transmits output signals 326 to output device 308 via I/O interface circuits (not shown). Memory circuit 320 includes three memory areas 328-332 including volatile and non-volatile memory. Memory area 328 stores the control program executed by processing circuit 318 and the fixed and variable data (e.g., calibration and empirical testing data) needed during execution. Optional memory area 330 stores an analyte map used by processing circuit 318 to identify the particular analyte being tested for at each micro-location 142 in array 104. When the map is present, processing circuit 318 may be programmed to identify analytes detected in the fluid sample by correlating the received data output signals 118 to the analytes identified in the map, and to generate output signals 326 to identify the detected analytes on output device 308. Memory area 332 stores a data acquisition array used by processing circuit 318 to accumulate the comparator positive transitions for each pixel element during the integration time period. The number of comparator positive transitions received during this period is indicative of the amount of light received by the photodetector 144 at each pixel element.

Input device 306 includes, for example, a keyboard, a mouse, a touch screen, or another input device for generating input signals 324 used to control operation of system 300. Input signals 324 from device 306 allow the user to, for example, start and stop operation of system 300, input analyte map data, input a desired integration time period, and input any other data or commands needed by processing circuit 318 to detect and identify analytes in the fluid sample being analyzed. Input signals 324 may also be used to configure computer 304 to read a

Processing circuit 318 then generates output signals 326 that, when applied to output device 308, causes output device 308 to display the corrected data. This corrected data is related to the presence and/or concentration of each analyte being tested for by relationships

5 determined empirically using known concentrations of analytes.

In another embodiment, the optional analyte map has been pre-programmed in memory area 330 with the identities of the analytes being tested for at each micro-location 142 in array 104 before the test is started (possibly by using input device 306). Then, instead of simply

10 outputting the corrected data for display on output device 308, processing circuit 318 performs the additional step of correlating the locations in data acquisition array 332 with the analyte map to identify the analytes, and generates output signals 326 to identify that analytes the corrected data represents.

15 In yet another embodiment, the data stored in memory area 328 includes threshold data indicative of the presence of each analyte in the fluid sample being analyzed. The threshold data for each analyte may have been determined by empirical testing using a fluid sample having a known minimum concentration of the analyte, or may simply be stored as
20 an offset from the dark readings. Processing circuit 318 then compares the corrected data to the threshold data (or the uncorrected data to the dark readings when offsets are used) to determine which analytes are present in the fluid sample being analyzed. Output signals 326 are then generated so that the analytes present in the fluid sample are displayed
25 or printed. This embodiment may also include the use of the analyte map to allow processing circuit 318 to identify the analytes whose presence in the sample fluid is detected.

In still another embodiment, the data within memory area 328 includes empirically-determined equations, curves or tables representing

116-122 and the surface of board 400 are protected from the fluid sample to be analyzed with an epoxy coating 408. Coating 408 is not applied over apertures 172 and 182 or die 140 to allow the fluid sample and the remaining components of the light-emitting reactions to be

5 applied to device 100.

Alternatively, multi-well chips are composed of three layers [see, e.g., Figures 8-11]. The bottom layer forms the lower section of each well and incorporates a semiconductor layer, a photodiode at the bottom of each well and an anode electrode, i.e., metal wire surrounding each 10 well. The middle layer fits into grooves in the bottom layer and is composed of a reflective metal layer, an insulating layer, preferably derivatized plastic or silicon, such as MYLAR (oriented polyethylene terephthalate is commercially available from the E.I. du Pont de Nemours & Co., Inc.) to which the specific antibody or ligand for each well is 15 attached [e.g., antibodies attached to MYLAR; see Figure 10]. The top cap layer forms the remaining upper portion of each well and also contains the cathode electrode. Analytes or reactants may be transported within or among wells by free field electrophoresis by supplying direct current, or by reversing the polarity of the current, 20 through the upper cathode and lower anode [e.g., see Figure 11].

When used, the chip is contacted with a sample and washed thoroughly. Buffer or other suitable compositions is added to each well, until the level is above the cathode position. The chip is then contacted with a composition containing a luciferin or, preferably a luciferase, 25 conjugated or fused or otherwise linked to an antibody or antibody binding portion thereof or a plurality of such fusions. The antibodies or portions thereof are each specific for the antigens of interest. The remaining components of the bioluminescence generating system are added and the chip is attached to a power source through a wire harness

preferred are silica substrates used in the fabrication of microelectric chip devices.

b. Fabrication Procedures

i. Microlithography

5 International patent application Publication Nos. WO 95/12808 and WO 96/07917 describe general microlithographic or photolithographic techniques that can be used for the fabrication of the complex "chip" type device that has a large number of small micro-locations. While the fabrication of devices does not require complex photolithography, the
10 selection of materials and the requirement that an electronic device function actively in aqueous solutions does not require special considerations.

The 64 micro-location device shown in Figure 14 of WO 95/12808 that can be fabricated using relatively simple mask design and standard
15 microlithographic techniques. Generally, the base substrate material would be a 1 to 2 centimeter square silicon wafer or a chip approximately 0.5 millimeter in thickness. The silicon chip is first overcoated with a 1 to 2 μm thick silicon dioxide (SiO_2) insulation coat, which is applied by plasma enhanced chemical vapor deposition
20 (PECVD).

The chips are preferably designed to contain detector elements, e.g., photodiodes, that are incorporated into the semiconductor layer and coupled through optical paths, such as by waveguides or other means, to the other optical paths of the chip. In preferred embodiments, the
25 detector element is comprised of a linear array of photodiodes with an approximate resolution of 1-5 microns, preferably 1-2 microns. Using a detector located with the chip, identification of a target in a test sample may be achieved at the site of the attachment of the biological molecule or anti-ligand.

because it has much less reactivity with the subsequent reagents used to modify the micro-electrode surfaces for the attachment of specific binding entities.

At this point the micro-electrode locations on the device are

- 5 modified with a specialized permeation and attachment layer, which is a crucial element required for the active functioning of the device. The objective is to create on the micro-electrode an intermediate permeation layer with selective diffusion properties and an attachment surface layer with optimal binding properties. The attachment layer should preferably
- 10 have from 10^5 to 10^7 functionalized locations per square micron (μm^2) for the optimal attachment of specific binding entities. The attachment of specific binding entities must not overcoat or insulate the surface to prevent the underlying micro-electrode from functioning. A functional device requires some fraction (~ 5% to 25%) of the actual metal electro-
- 15 electrode surface to remain accessible to solvent (H_2O) molecules, and to allow the diffusion of counter-ions (e.g., Na^+ and Cl^-) and electrolysis gases (e.g., O_2 and H_2) to occur.

The intermediate permeation layer must also allow diffusion to occur. Additionally, the permeation layer should have a pore limit

- 20 property that inhibits or impedes the larger binding entities, reactants, and analytes from physical contact with the micro-electrode surface. The permeation layer keeps the active micro-electrode surface physically distinct from the binding entity layer of the micro-location.

- 25 In terms of the primary device function, this design allows the electrolysis reactions required for electrophoretic transport to occur on micro-electrode surface, but avoids adverse electrochemical effects to the binding entities, reactants, and analytes.

One preferred procedure for the derivatization of the metal micro-electrode surface uses aminopropyltriethoxy silane (APS). APS reacts

case of micromachined devices, connective circuitry and large electrode structures can be printed onto materials using standard circuit board printing techniques known to those skilled in the art.

In the instant application, the chips are preferably designed to

- 5 contain detector elements, e.g., photodiodes, that are incorporated into the semiconductor layer and coupled through optical paths, such as by waveguides or other means, to the other optical paths of the chip. In preferred embodiments, the detector element is comprised of a linear array of photodiodes with an approximate resolution of 1-5 microns,
- 10 preferably 1-2 microns. Using a detector located with the chip, identification of a target in a test sample may be achieved at the site of the attachment of the biological molecule or anti-ligand.

Addressable micro-location devices can be fabricated relatively easily using micro-machining techniques. Figure 15 of WO 95/12808

- 15 shows a schematic of a representative 96 micro-location device. This micro-location device is fabricated from a suitable material stock (2 cm x 4 cm x 1 cm), by drilling 96 proportionately spaced holes (1 mm in diameter) through the material. An electrode circuit board is formed on a thin sheet of plastic material stock, which fit precisely over the top of the
- 20 micro-location component. The underside of the circuit board contains the individual wires (printed circuit) to each micro-location. Short platinum electrode structures (~ 3-34 mm) are designed to extend down into the individual micro-location chambers. The printed circuit wiring is coated with a suitable water-proof insulating material. The printed circuit
- 25 wiring converges to a socket, which allows connection to a multiplex switch controller and DC power supply. The device is partially immersed and operates in a common buffer reservoir.

While the primary function of the micro-locations in devices fabricated by micro-machining and microlithography techniques is the

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The above description for the design and fabrication of a device should not be considered as a limit to other variations or forms of the basic device. Many variations of the device with larger or smaller numbers of addressable micro-locations are envisioned for different

5 analytical and preparative applications. Variations of the device with larger addressable locations are envisioned for preparative biopolymer synthesis applications. Variations are also contemplated as electronically addressable and controllable reagent dispensers for use with other devices, including those produced by microlithographic techniques.

10 c. Self-addressing of chips

The chips and devices described in International patent application Publication Nos. WO 95/12808 and WO 96/07917 are able to electronically self-address each micro-location with a specific binding entity. The device itself directly affects or causes the transport and 15 attachment of specific binding entities to specific micro-locations. The device self-assembles itself in the sense that no outside process, mechanism, or equipment is needed to physically direct, position, or place a specific binding entity at a specific micro-location. This self-addressing process is rapid and specific, and can be carried out in either 20 a serial or parallel manner.

A device can be serially addressed with specific binding entities by maintaining the selected micro-location in a DC mode and at the opposite charge (potential) to that of a specific binding entity. All other micro-locations are maintained at the same charge as the specific binding 25 entity. In cases where the binding entity is not in excess of the attachment sites on the micro-location, it is necessary to activate only one other micro-electrode to affect the electrophoretic transport to the specific micro-location. The specific binding entity is rapidly transported (in a few seconds, or preferably less than a second) through the solution,

Chromatography, Advances in Experimental Medicine and Biology, vol. 42, ed. R. Dunlap, Plenum Press, N.Y. (1974); U.S. Patent No. 5,451,683, see, also U.S. Patent Nos. 5,624,711, 5,412,087, 5,679,773, 5,143,854], particularly silicon chips are known.

5 These methods typically involve derivatization of the solid support to form a uniform layer of reactive groups on the support surface and subsequent attachment of the biological molecule to the derivatized surface via a covalent bond between the reactive group and a reactive moiety present on the biological molecule. Presently preferred methods 10 are those applicable for the derivatization and attachment of biological molecules to silica substrates, particularly methods for derivatizing the silica surface of microelectronic chip devices.

a. **Derivatization of silica substrates**

Numerous methods for derivitizing silica surfaces or for coating 15 surfaces with silica and then derivatizing the surface are known. A number of reagents may be used to derivatize the surface of a silica substrate. For example, U.S. Pat. No. 4,681,870 describes a method for introducing free amino or carboxyl groups onto a silica matrix. Alternatively, a layer of free amino groups or carboxyl groups may be 20 introduced using amino- and carboxymethyl silane derivatives, such as 3-aminopropyltriethoxysilane, 3-aminopropyltrimethoxysilane, 4-amino-butyltriethoxysilane, (aminoethylaminomethyl)phenethyltrimethoxysilane, 2-(carbomethoxy)ethyltrichlorosilane, (10-carbomethoxydecyl)dimethyl-chlorosilane and 2-(carbomethoxy)ethylmethyldichlorosilane (e.g., see 25 Hulls Catalog).

The silica surface may also be derivatized to introduce a layer of hydroxyl groups using alkyl- and alkoxyalkyl halogenated silane derivatives. The alkoxy groups of trialkoxysilanes are hydrolyzed to their corresponding silanol species, which may occur during the formal

a biological molecule, such as a protein, a protein nucleic acid or other anti-ligand, in the presence of a carbodiimide. The use of carbodiimides [e.g., *N*-ethyl-*N'*-(γ -dimethylaminopropyl)carbodiimide], as coupling agents is well known to those of skill in the art [see, e.g., Bodansky *et al.* in

5 "The Practice of Peptide Synthesis," Springer-Verlag, Berlin (1984)].

Another method for attaching biological molecules involves modification of a silica surface through the successive application of multiple layers of biotin, avidin and extenders [see, e.g., U.S. Patent No. 4,282,287]; other methods involve photoactivation in which a

10 polypeptide chain is attached to a solid substrate by incorporating a light-sensitive unnatural amino acid group into the polypeptide chain and exposing the product to low-energy ultraviolet light [see, e.g., U.S.

Patent No. 4,762,881]. Oligonucleotides have also been attached using a photochemically active reagents, such as a psoralen compound, and a

15 coupling agent, which attaches the photoreagent to the substrate [see, e.g., U.S. Patent No. 4,542,102 and U.S. Patent No. 4,562,157].

Similar methods are applicable to peptide nucleic acids. Photoactivation of the photoreagent binds a nucleic acid molecule or peptide nucleic acid molecule to the substrate to give a surface-bound probe. In certain

20 embodiments, the photoactivation may occur *in situ* by selecting an appropriate bioluminescence generating system with an appropriate emission wavelength sufficient to photoactivate and immobilize the nucleic acid.

Furthermore, U.S. Pat. No. 5,451,683 describes a technique for

25 attaching biochemical ligands to surfaces of matrices by attachment of a photoactivatable biotin derivatives. Photolytic activation of the biotin derivatives for biotin analogs having strong binding affinity for avidin or streptavidin. The biotinylated ligands are immobilized on activated regions previously treated with avidin or streptavidin.

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silane-alcohol solution. After this treatment, the chip is washed using large amounts of absolute ethanol, followed by washes in THF or dioxane, hexane (ultrapure) and finally pentane, which is evaporated under a stream of dry nitrogen.

5 The efficiency of the derivatization of the surface of the chip may be determined by coupling an appropriate fluorescent amine (carboxyl derivatized) or fluorescent carboxylic acid (amino derivatized) to the surface of the chip by exciting the fluorescence of the bound molecules using a laser of appropriate wavelength. Appropriate compounds for this
10 purpose may be amino, carboxyl or other reactive derivatives of fluorescein, rhodamine or Texas Red, which are known to those of skill in the art and are also commercially available (e.g., see Molecular Probes, Inc.).

15 The isothiocyanates of fluorescein, rhodamine, or Texas Red, for example, react in an irreversible and covalent manner with any free amino groups on the silica surface. A solution of an effective concentration of fluorescein (about 10 mM) isothiocyanate (mixed isomers) in acetone or dioxane is placed on the amine-derivatized silica of the chip for sufficient time, typically about 30 minutes at ambient
20 temperatures. To remove all unreacted material, the chip is washed with hot (i.e., 60 °C) solutions of acetone, hexane and pentane or other suitable solvent. A region on the same chip that has not been chemically derivatized is similarly treated with the fluorescein isothiocyanate as a control. A small amount of direct covalent reaction with the glass is
25 possible and thus the control should be performed to indicate background levels. The fluorescence of the bound fluorescein can be excited using a suitable sources, such as an argon ion laser (e.g., 488 nm), preferably using a 45-degree angle geometry. The argon laser can further contain a photomultiplier equipped with a 10 nm bandpass filter for detecting the

exposure to UV or visible light. In some embodiments, several linkers may be included in order to take advantage of desired properties of each linker.

Chemical linkers and peptide linkers may be inserted by covalently

5 coupling the linker to the anti ligand and to the surface of the chip. The heterobifunctional agents, described below, may be used to effect such covalent coupling. Peptide linkers may also be linked by expressing DNA encoding the linker and the anti ligand, e.g., an antibody, as a fusion protein.

10 Numerous heterobifunctional cross-linking reagents that are used to form covalent bonds between amino groups and thiol groups and to introduce thiol groups into proteins, are known to those of skill in this art (see, e.g., the PIERCE CATALOG, ImmunoTechnology Catalog & Handbook, 1992-1993, which describes the preparation of and use of

15 such reagents and provides a commercial source for such reagents; see, also, e.g., Cumber et al. (1992) Bioconjugate Chem. 3:397-401; Thorpe et al. (1987) Cancer Res. 47:5924-5931; Gordon et al. (1987) Proc. Natl. Acad. Sci. 84:308-312; Walden et al. (1986) J. Mol. Cell Immunol. 2:191-197; Carlsson et al. (1978) Biochem. J. 173: 723-737; Mahan et al. (1987) Anal. Biochem. 162:163-170; Wawryznaczak et al. (1992) Br. J. Cancer 66:361-366; Fattom et al. (1992) Infection & Immun. 60:584-589). These reagents may be used to form covalent bonds between the anti ligand and the luciferase molecule. These reagents include, but are not limited to: N-succinimidyl-3-(2-pyridyldithio)-

20 propionate (SPDP; disulfide linker); sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP); succinimidyl-oxycarbonyl- α -methyl benzyl thiosulfate (SMBT, hindered disulfate linker); succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexanoate (LC-SPDP); sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate

25

3. Nucleic acid and peptide nucleic acid conjugates

The luciferase molecules described herein may also be conjugated to nucleic acids or peptide nucleic acids. The coupling may also be effected in the absence or presence of a linker. Methods for conjugating

- 5 nucleic acids, at the 5'ends, 3' ends and elsewhere, to the amino and carboxyl terminii and other sites in proteins are known to those of skill in the art (for a review see e.g., Goodchild, (1993) In:Perspectives in Bioconjugate Chemistry, Mears, Ed., American Chemical Society, Washington, D.C. pp.77-99. For example, proteins have been linked to
- 10 nucleic acids using ultraviolet irradiation (Sperling et al. (1978) Nucleic Acids Res. 5:2755-2773; Fiser et al. (1975) FEBS Lett. 52:281-283), bifunctional chemicals (Bäumert et al. (1978) Eur. J. Biochem. 89353-359; and Oste et al. (1979) Mol. Gen. Genet. 168::81-86) photochemical cross-linking (Vainin et al. (1981) FEBS Lett. 124:89-92; Rinke et al.
- 15 (1980) J.Mol.Biol. 137:301-314; Millon et al. (1980) Eur. J. Biochem. 110:485-454).

In addition, the carboxyl terminus of a luciferase may be conjugated to one of the free amino groups of peptide nucleic acids [e.g., see Nielsen et al. (1990) Science 254:1497-1500; Peffer et al. (1993)

- 20 Proc. Natl. Acad. Sci. U.S.A. 90:10648-10652) using standard carbodiimide peptide chemistry.

Additional sites for conjugation can also be introduced into the nucleic acid molecule by chemical modification of one or more position or by the introduction of a small antigenic determinant covalently coupled to

- 25 the 5' or 3'-end of the molecule. A variety of small antigenic determinants (i.e., His Tags, flg antigens, S-Tags, dioxigenin and the like) are known to those of skill in the art and are also commercially available [e.g., Boehringer Mannheim, Indianapolis, IN; Novagen, Inc., Madison WI]. Modified nucleic acids and peptide nucleic acid analogs may also be

proteins known as frustulins [see, e.g., Kroger *et al.* (1996) Eur. J.

Biochem. 239:259-264]. In marine diatoms, new valves are produced after cell division and cytokinesis of the mother protoplast.

The resulting daughter protoplast produces a new valve in a specialized

5 intracellular organelle, the silica deposition vesicle. Silica is transported into the silicalemma where nucleation and epitaxial growth of Si monomers occurs on a template or more complex polymerization of silica occurs within the vesicles [see, e.g., Pickett-Heaps *et al.* (1979) Bio. Cell. 35:199-203; Sullivan (1986) Ciba Found. Symp. 121:59-89;

10 Pickett-Heaps *et al.* (1990) Prog. Phycol. Res. 7:1-186].

In radiolaria, the deposition of the silicate skeleton is associated with a cytoplasmic sheath that encloses, molds and deposits the skeleton termed "cytokalymma". The thickness of the skeleton may be influenced by the physiological state of the organism. The cytokalymma may

15 function in an analogous manner to the silicalemma in the silica deposition in diatoms.

Artificial inorganic assemblies that mimic diatom and radiolaria exoskeletons have been described [e.g., see Oliver *et al.* (1995) Nature 378:47-50; U.S. Pat. Nos. 5,057,296, 5,108,725 and 5,364,797].

20 Several morphologies of mesophases may be formed, e.g., lamellar, hexagonal and cubic mesostructures, depending on the selected starting materials and conditions used. These crystalline mesostructures, however, may only be formed at higher temperatures, which may be unsuitable for use with certain matrix materials.

25 Models have been proposed to explain the biomineralization process and also the formation and morphology of these surfactant-silicate mesostructures [see, e.g., Sullivan (Monnier *et al.* Science 261:1299-1303)]. For example, it is postulated that the control of the silicate wall thickness is related to the double layer potential: silicate

F. Methods of use

1. Immunoassays

The chips described herein may be used in diagnostic assays. For example, the chips are used in an immunoassay for the

- 5 detection of infectious agents using antibodies directed against infectious microorganisms, e.g., bacteria, viruses, protozoa and other lower eukaryotic organisms [e.g., see Figure 20]. A plurality of anti-ligands, e.g., antibodies, is linked to each location or microlocation on the chip or attached to an appropriate layer of the reflective middle layer of a multi-
- 10 well chip creating a panel of antibodies raised against a particular microorganisms. The antibody-bound chip is placed directly into a sample of body fluid obtained from a patient, e.g., urine, sputum or blood.

Sufficient time is allowed to form antibody-antigen complexes and

- 15 the chip is removed and rinsed thoroughly. A solution containing a plurality of secondary antibodies directed against a panel of known pathogens conjugated to a luciferase or luciferase fusion protein is added, which may be directed against the same antigen or another antigen present on the targeted species. Alternatively, a phage or virus
- 20 may be employed that has been genetically engineered to contain DNA encoding a luciferase. Preferably, the virus or phage has a broad specificity.

The chip or individual well is washed, and the remaining components of the bioluminescence generating system, e.g., a luciferin

- 25 and any necessary activators, are added. If an antigen has been detected, light is emitted from the bound luciferase, which is in turn detected by the photodiodes located within the semiconductor layer in the attached chip. In the multi-well chip system, the output signal of the bioluminescent reaction is increased by detecting light directly emitted

detected by the addition of an anti ligand conjugated to a component of a bioluminescence generating system, preferably a luciferase. Presently preferred anti ligands are antibodies, or a F(Ab)₂ fragments thereof, that preferentially recognize double stranded nucleic acids or the associated 5 small antigenic determinant. Antibodies that recognize double stranded DNA are associated with a number of autoimmune diseases [e.g., see Tsuzaka *et al.* (1996) Clin. Exp. Immunol. **106**:504-508; Kanda *et al.* (1997) Arthritis Rheum. **40**:1703-1711].

The chip or individual well is washed to remove unbound antibody 10 - luciferase conjugate, and the remaining components of the bioluminescence generating system, e.g., a luciferin and any necessary activators, are added. If a complementary nucleic acid or peptide nucleic acid has been detected, light is emitted from the bound luciferase, which is in turn detected by the photodiodes located within the semiconductor 15 layer in the attached chip.

The assay may also be used quantitatively by adding a known amount of luciferin to the well and by measuring the rate of the utilization of the luciferin (i.e., a reduction in light production over time is proportional to the amount present in the sample as compared to 20 controls).

3. Detection of antibiotic sensitivity

Among other uses for the chip is testing the sensitivity of a clinical isolate to known antibiotics or as a device to screen for antibacterial agents. For example, after detecting light emission from a targeted well, 25 an isolate may be grown directly in the well for a short period by the addition of a suitable growth medium [e.g., L-broth or other undefined medium] followed by incubation under appropriate environmental conditions, such as temperatures of 20°C to 42°C under aerobic or anaerobic atmospheres.

The silicon-synapse electrodes may be permanently implanted in an afflicted patient by insertion into the appropriate stereotaxic locations in the spinal cord by MRI localization [see Figure 19]. To implant the electrodes, microholes are drilled into the spinal cord using a suitable

5 laser, such as a CO₂ laser, and the electrode is placed into proximity of a known nerve fiber or bundle. The placement of the silicon-synapse may be from superficial to deep within the spinal cord along known neuronal pathways. Exact tracing of the appropriate neuron is preferable, though not essential, because the human brain will reprogram itself to send the

10 signal along those neurons that transmit the proper signal.

The transmission of neuronal impulses involves various neurotransmitters, such as acetylcholine, which are released into the synapse. Upon binding of the ligand to the enzyme, such as the binding of acetylcholine to the esterase, the linked luciferase is, if previously

15 inactive, is activated by the binding, or if previously active, is inactivated by the binding [see Figure 18]. In the presence of the remaining components of a bioluminescence generating system, light is produced (or is quenched), which change is detected by the photodiodes associated with the chip. This detection generates one or more electrical

20 or data signals that is/are sent through one or more wires leading to a computer, such a miniature computer that is attached to a belt, which processes the information. The processed information is transmitted by appropriate means, such as a fiber, to one or more electrodes, which are attached to any desired device or effector, particularly a muscle. Upon

25 receipt of the signal, work, such as a muscle twitch, occurs and body movements may be initiated. The devices will be inserted in a manner that bypasses a lesioned area of the spinal cord [see, e.g., see Figure 17].

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Summary of Sequences of Representative luciferases and the reductase set forth in the Sequence Listing

1. SEQ ID NO. 1 *Renilla reinformis* Luciferase [U.S. Patent No. 5,418,155]
2. SEQ ID NO. 2 *Cypridina hilgendorfii* luciferase [EP 0 387 355]
- 5 3. SEQ ID NO. 3 Modified *Luciola cruciata* Luciferase [firefly; U.S. Patent No. 4,968,613]
4. SEQ ID NO. 4 *Vargula (Cypridina)* luciferase [Thompson et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:6567-6571 and from JP 3-30678 Osaka
- 10 5. SEQ ID NO. 5 Apoaequorin-encoding gene [U.S. Patent No. 5,093,240, pAQ440]
6. SEQ ID NO. 6 Recombinant *Aequorin* AEQ1 [Prasher et al. (1987) "Sequence Comparisons of cDNAs Encoding for Aequorin Isotypes," Biochemistry 26:1326-1332]
- 15 7. SEQ ID NO. 7 Recombinant *Aequorin* AEQ2 [Prasher et al. (1987)]
8. SEQ ID NO. 8 Recombinant *Aequorin* AEQ3 [Prasher et al. (1987)]
9. SEQ ID NO. 9 *Aequorin* photoprotein [Charbonneau et al. (1985) "Amino Acid Sequence of the Calcium-Dependent Photoprotein Aequorin," Biochemistry 24:6762-6771]
- 20 10. SEQ ID NO. 10 *Aequorin* mutant with increased bioluminescence activity [U.S. Patent No. 5,360,728; Asp 124 changed to Ser]
11. SEQ ID NO. 11 *Aequorin* mutant with increased bioluminescence activity [U.S. Patent No. 5,360,728; Glu 135 changed to Ser]
12. SEQ ID NO. 12 *Aequorin* mutant with increased bioluminescence
- 25 activity [U.S. Patent No. 5,360,728 Gly 129 changed to Ala]
13. SEQ ID NO. 13 Recombinant apoaequorin [sold by Sealite, Sciences, Bogart, GA as AQUALITE®, when reconstituted to form aequorin]
14. SEQ ID NO. 14 *Vibrio fisheri* Flavin reductase [U.S. Patent No. 5,484,723]

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(vi) ORIGINAL SOURCE:

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...942
- (D) OTHER INFORMATION: *Renilla Reiniformis Luciferase*

(x) PUBLICATION INFORMATION:

PATENT NO.: 5,418,155

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGC TTA AAG ATG ACT TCG AAA GTT TAT GAT CCA GAA CAA AGG AAA CGG Ser Leu Lys Met Thr Ser Lys Val Tyr Asp Pro Glu Gln Arg Lys Arg	48
1 5 10 15	
ATG ATA ACT GGT CCG CAG TGG TGG GCC AGA TGT AAA CAA ATG AAT GTT Met Ile Thr Gly Pro Gln Trp Trp Ala Arg Cys Lys Gln Met Asn Val	96
20 25 30	
CTT GAT TCA TTT ATT AAT TAT TAT GAT TCA GAA AAA CAT GCA GAA AAT Leu Asp Ser Phe Ile Asn Tyr Tyr Asp Ser Glu Lys His Ala Glu Asn	144
35 40 45	
GCT GTT ATT TTT TTA CAT GGT AAC GCG GCC TCT TCT TAT TTA TGG CGA Ala Val Ile Phe Leu His Gly Asn Ala Ala Ser Ser Tyr Leu Trp Arg	192
50 55 60	
CAT GTT GTG CCA CAT ATT GAG CCA GTA GCG CGG TGT ATT ATA CCA GAT His Val Val Pro His Ile Glu Pro Val Ala Arg Cys Ile Ile Pro Asp	240
65 70 75 80	
CTT ATT GGT ATG GGC AAA TCA GGC AAA TCT GGT AAT GGT TCT TAT AGG Leu Ile Gly Met Gly Lys Ser Gly Lys Ser Gly Asn Gly Ser Tyr Arg	288
85 90 95	
TTA CTT GAT CAT TAC AAA TAT CTT ACT GCA TGG TTG AAC TTC TTA ATT Leu Leu Asp His Tyr Lys Tyr Leu Thr Ala Trp Leu Asn Phe Leu Ile	336
100 105 110	
TAC CAA AGA AGA TCA TTT TTT GTC GGC CAT GAT TGG GGT GCT TGT TTG Tyr Gln Arg Arg Ser Phe Phe Val Gly His Asp Trp Gly Ala Cys Leu	384
115 120 125	
GCA TTT CAT TAT AGC TAT GAG CAT CAA GAT AAG ATC AAA GCA ATA GTT Ala Phe His Tyr Ser Tyr Glu His Gln Asp Lys Ile Lys Ala Ile Val	432
130 135 140	
CAC GCT GAA AGT GTA GTA GAT GTG ATT GAA TCA TGG GAT GAA TGG CCT His Ala Glu Ser Val Val Asp Val Ile Glu Ser Trp Asp Glu Trp Pro	480
145 150 155 160	
GAT ATT GAA GAA GAT ATT GCG TTG ATC AAA TCT GAA GAA GGA GAA AAA Asp Ile Glu Glu Asp Ile Ala Leu Ile Lys Ser Glu Glu Gly Glu Lys	528
165 170 175	
ATG GTT TTG GAG AAT AAC TTC TTC GTG GAA ACC ATG TTG CCA TCA AAA Met Val Leu Glu Asn Asn Phe Phe Val Glu Thr Met Leu Pro Ser Lys	576
180 185 190	

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1	5	10	15	
AAC TGC CAG GAT GCA TGT CCT GTC GAA GCT GAA GCA CCG TCA AGT ACA				96
Asn Cys Gln Asp Ala Cys Pro Val Glu Ala Glu Ala Pro Ser Ser Thr				
20	25	30		
CCA ACA GTC CCA ACA TCT TGT GAA GCT AAA GAA GGA GAA TGT ATC GAT				144
Pro Thr Val Pro Thr Ser Cys Glu Ala Lys Glu Gly Glu Cys Ile Asp				
35	40	45		
ACC AGA TGC GCA ACA TGT AAA CGA GAC ATA CTA TCA GAC GGA CTG TGT				192
Thr Arg Cys Ala Thr Cys Lys Arg Asp Ile Leu Ser Asp Gly Leu Cys				
50	55	60		
GAA AAT AAA CCA GGG AAG ACA TGC TGT AGA ATG TGC CAG TAT GTA ATT				240
Glu Asn Lys Pro Gly Lys Thr Cys Cys Arg Met Cys Gln Tyr Val Ile				
65	70	75	80	
GAA TCC AGA GTA GAA GCT GGT GGA TAT TTT AGA ACG TTT TAC GCC AAA				288
Glu Ser Arg Val Glu Ala Ala Gly Tyr Phe Arg Thr Phe Tyr Ala Lys				
85	90	95		
AGA TTT AAT TTT CAG GAA CCT GGT AAA TAT GTG CTG GCT CGA GGA ACC				336
Arg Phe Asn Phe Gln Glu Pro Gly Lys Tyr Val Leu Ala Arg Gly Thr				
100	105	110		
AAG GGT GGC GAC TGG TCT GTA ACC CTC ACC ATG GAG AAT CTA GAT GGA				384
Lys Gly Asp Trp Ser Val Thr Leu Thr Met Glu Asn Leu Asp Gly				
115	120	125		
CAG AAG GGA GCT GTA CTG ACT AAG ACA ACA CTG GAG GTA GTA GGA GAC				432
Gln Lys Gly Ala Val Leu Thr Lys Thr Leu Glu Val Val Gly Asp				
130	135	140		
GTA ATA GAC ATT ACT CAA GCT ACT GCA GAT CCT ATC ACA GTT AAC GGA				480
Val Ile Asp Ile Thr Gln Ala Thr Ala Asp Pro Ile Thr Val Asn Gly				
145	150	155	160	
GGA GCT GAC CCA GTT ATC GCT AAC CCG TTC ACA ATT GGT GAG GTG ACC				528
Gly Ala Asp Pro Val Ile Ala Asn Pro Phe Thr Ile Gly Glu Val Thr				
165	170	175		
ATT GCT GTT GTC GAA ATA CCC GGC TTC AAT ATT ACA GTC ATC GAA TTC				576
Ile Ala Val Val Glu Ile Pro Gly Phe Asn Ile Thr Val Ile Glu Phe				
180	185	190		
TTT AAA CTA ATC GTG ATA GAT ATT CTG GGA GGA AGA TCT GTG AGA ATT				624
Phe Lys Leu Ile Val Ile Asp Ile Leu Gly Gly Arg Ser Val Arg Ile				
195	200	205		
GCT CCA GAC ACA GCA AAC AAA GGA CTG ATA TCT GGT ATC TGT GGT AAT				672
Ala Pro Asp Thr Ala Asn Lys Gly Leu Ile Ser Gly Ile Cys Gly Asn				
210	215	220		
CTG GAG ATG AAT GAC GCT GAT GAC TTT ACT ACA GAC GCA GAT CAG CTG				720
Leu Glu Met Asn Asp Ala Asp Phe Thr Thr Asp Ala Asp Gln Leu				
225	230	235	240	
GCG ATC CAA CCC AAC ATA AAC AAA GAG TTC GAC GGC TGC CCA TTC TAC				768
Ala Ile Gln Pro Asn Ile Asn Lys Glu Phe Asp Gly Cys Pro Phe Tyr				
245	250	255		

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Phe Asp Ser Ser Ile Asp Glu Lys Cys Asn Val Cys Tyr Lys Pro Asp	
500 505 510	
CGT ATT GCA CGA TGT ATG TAC GAG TAT TGC CTG AGG GGA CAG CAA GGA	1584
Arg Ile Ala Arg Cys Met Tyr Glu Tyr Cys Leu Arg Gly Gln Gin Gly	
515 520 525	
TTC TGT GAC CAT GCT TGG GAG TTC AAA AAA GAA TGC TAC ATA AAG CAT	1632
Phe Cys Asp His Ala Trp Glu Phe Lys Lys Glu Cys Tyr Ile Lys His	
530 535 540	
GGA GAC ACT CTA GAA GTA CCA CCT GAA TGC CAA TAA ATGAACAAAG	1678
Gly Asp Thr Leu Glu Val Pro Pro Glu Cys Gln	
545 550 555	
ATACAGAAC TAAGACTACT ACAGCAGAAC ATAAAAAGAGA AGCTGTAGTT CTTCAAAAC	1738
AGTATATTTT GATGTACTCA TTGTTTACTT ACATAAAAAT AAATTGTTAT TATCATAACG	1798
TAAAGAAAAA AAAAAAAA AAAA	1822

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1644 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...1644
- (D) OTHER INFORMATION: Luciola Cruciata Luciferase (Firefly)

(x) PUBLICATION INFORMATION:

PATENT NO.: 4,968,613

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG GAA AAC ATG GAA AAC GAT GAA AAT ATT GTA GTT GGA CCT AAA CCG	48
Met Glu Asn Met Glu Asn Asp Glu Asn Ile Val Val Gly Pro Lys Pro	
1 5 10 15	
TTT TAC CCT ATC GAA GAG GGA TCT GCT GGA ACA CAA TTA CGC AAA TAC	96
Phe Tyr Pro Ile Glu Glu Gly Ser Ala Gly Thr Gln Leu Arg Lys Tyr	
20 25 30	
ATG GAG CGA TAT GCA AAA CTT GGC GCA ATT GCT TTT ACA AAT GCA GTT	144
Met Glu Arg Tyr Ala Lys Leu Gly Ala Ile Ala Phe Thr Asn Ala Val	
35 40 45	
ACT GGT GTT GAT TAT TCT TAC GCC GAA TAC TTG GAG AAA TCA TGT TGT	192
Thr Gly Val Asp Tyr Ser Tyr Ala Glu Tyr Leu Glu Lys Ser Cys Cys	
50 55 60	
CTA GGA AAA GCT TTG CAA AAT TAT GGT TTG GTT GAT GGC AGA ATT	240
Leu Gly Lys Ala Leu Gln Asn Tyr Gly Leu Val Val Asp Gly Arg Ile	
65 70 75 80	

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Leu Ser Lys Glu Val Gly Glu Ala Val Ala Arg Arg Phe Asn Leu Pro			
325	330	335	
GGT GTT CGT CAA GGT TAT GGT TTA ACA GAA ACA ACA TCT GCC ATT ATT			1056
Gly Val Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Ile			
340	345	350	
ATT ACA CCA GAA GGA GAC GAT AAA CCA GGA GCT TCT GGA AAA GTC GTG			1104
Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Ser Gly Lys Val Val			
355	360	365	
CCG TTG TTT AAA GCA AAA GTT ATT GAT CTT GAT ACC AAA AAA TCT TTA			1152
Pro Leu Phe Lys Ala Lys Val Ile Asp Leu Asp Thr Lys Lys Ser Leu			
370	375	380	
GGT CCT AAC AGA CGT GGA GAA GTT TGT GTT AAA GGA CCT ATG CTT ATG			1200
Gly Pro Asn Arg Arg Gly Glu Val Cys Val Lys Gly Pro Met Leu Met			
385	390	395	400
AAA GGT TAT GTA AAT AAT CCA GAA GCA ACA AAA GAA CTT ATT GAC GAA			1248
Lys Gly Tyr Val Asn Asn Pro Glu Ala Thr Lys Glu Leu Ile Asp Glu			
405	410	415	
GAA GGT TGG CTG CAC ACC GGA GAT ATT GGA TAT TAT GAT GAA GAA AAA			1296
Glu Gly Trp Leu His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu Glu Lys			
420	425	430	
CAT TTC TTT ATT GTC GAT CGT TTG AAG TCT TTA ATC AAA TAC AAA GGA			1344
His Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly			
435	440	445	
TAC CAA GTA CCA CCT GCC GAA TTA GAA TCC GTT CTT TTG CAA CAT CCA			1392
Tyr Gln Val Pro Pro Ala Glu Leu Glu Ser Val Leu Leu Gln His Pro			
450	455	460	
TCT ATC TTT GAT GCT GGT GTT GCC GGC GTT CCT GAT CCT GTA GCT GGC			1440
Ser Ile Phe Asp Ala Gly Val Ala Gly Val Pro Asp Pro Val Ala Gly			
465	470	475	480
GAG CTT CCA GGA GCC GTT GTT GTA CTG GAA AGC GGA AAA AAT ATG ACC			1488
Glu Leu Pro GLy Ala Val Val Val Leu Glu Ser Gly Lys Asn Met Thr			
485	490	495	
GAA AAA GAA GTA ATG GAT TAT GTT GCA AGT CAA GTT TCA AAT GCA AAA			1536
Glu Lys Glu Val Met Asp Tyr Val Als Ser Gln Val Ser Asn Ala Lys			
500	505	510	
CGT TTA CGT GGT GGT GTT CGT TTT GTG GAT GAA GTA CCT AAA GGT CTT			1584
Arg Leu Arg Gly Gly Val Arg Phe Val Asp Glu Val Pro Lys Gly Leu			
515	520	525	
ACT GGA AAA ATT GAC GGC AGA GCA ATT AGA GAA ATC CTT AAG AAA CCA			1632
Thr Gly Lys Ile Asp Gly Arg Ala Ile Arg Glu Ile Leu Lys Lys Pro			
530	535	540	
GTT GCT AAG ATG			1644
Val Ala Lys Met			
545			

(2) INFORMATION FOR SEQ ID NO:4:

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145	150	155	160	
GGA GCT GAC CCA GTT ATC GCT AAC CCG TTC ACA ATT GGT GAG GTG ACC				528
Gly Ala Asp Pro Val Ile Ala Asn Pro Phe Thr Ile Gly Glu Val Thr				
165		170	175	
ATT GCT GTT GTT GAA ATA CCG GGC TTC AAT ATC ACA GTC ATC GAA TTC				576
Ile Ala Val Val Glu Ile Pro Gly Phe Asn Ile Thr Val Ile Glu Phe				
180		185	190	
TTT AAA CTA ATC GTG ATT GAT ATT CTG GGA GGA AGA TCT GTC AGA ATT				624
Phe Lys Leu Ile Val Ile Asp Ile Leu Gly Gly Arg Ser Val Arg Ile				
195		200	205	
GCT CCA GAC ACA GCA AAC AAA GGA CTG ATA TCT GGT ATC TGT GGT AAT				672
Ala Pro Asp Thr Ala Asn Lys Gly Leu Ile Ser Gly Ile Cys Gly Asn				
210		215	220	
CTG GAG ATG AAT GAC GCT GAT GAC TTT ACT ACA GAT GCA GAT CAG CTG				720
Leu Glu Met Asn Asp Ala Asp Asp Phe Thr Thr Asp Ala Asp Gln Leu				
225		230	235	240
GCG ATC CAA CCC AAC ATA AAC AAA GAG TTC GAC GGC TGC CCA TTC TAT				768
Ala Ile Gln Pro Asn Ile Asn Lys Glu Phe Asp Gly Cys Pro Phe Tyr				
245		250	255	
GGC AAT CCT TCT GAT ATC GAA TAC TGC AAA GGT CTG ATG GAG CCA TAC				816
Gly Asn Pro Ser Asp Ile Glu Tyr Cys Lys Gly Leu Met Glu Pro Tyr				
260		265	270	
AGA GCT GTA TGT CGT AAC AAT ATC AAC TTC TAC TAT TAC ACT CTA TCC				864
Arg Ala Val Cys Arg Asn Asn Ile Asn Phe Tyr Tyr Tyr Thr Leu Ser				
275		280	285	
TGT GCC TTC GCT TAC TGT ATG GGA GGA GAA GAA AGA GCT AAA CAC GTC				912
Cys Ala Phe Ala Tyr Cys Met Gly Gly Glu Glu Arg Ala Lys His Val				
290		295	300	
CTT TTC GAC TAT GTT GAG ACA TGC GCT GCG CCG GAA ACG AGA GGA ACG				960
Leu Phe Asp Tyr Val Glu Thr Cys Ala Ala Pro Glu Thr Arg Gly Thr				
305		310	315	320
TGT GTT TTA TCA GGA CAT ACT TTC TAT GAC ACA TTC GAC AAA GCA AGA				1008
Cys Val Leu Ser Gly His Thr Phe Tyr Asp Thr Phe Asp Lys Ala Arg				
325		330	335	
TAT CAA TTC CAG GGC CCA TGC AAG GAG ATT CTG ATG GCC GCA GAC TGT				1056
Tyr Gln Phe Gly Pro Cys Lys Glu Ile Leu Met Ala Ala Asp Cys				
340		345	350	
TAC TGG AAC ACA TGG GAT GTA AAG GTT TCA CAT AGA GAC GTC GAA TCA				1104
Tyr Trp Asn Thr Trp Asp Val Lys Val Ser His Arg Asp Val Glu Ser				
355		360	365	
TAC ACT GAG GTA GAG AAA GTA ACA ATC AGG AAA CAG TCA ACT GTA GTA				1152
Tyr Thr Glu Val Glu Lys Val Thr Ile Arg Lys Gln Ser Thr Val Val				
370		375	380	
GAT CTC ATT GTG GAT GGC AAG CAG GTC AAG GTT GGA GGA GTG GAT GTA				1200
Asp Leu Ile Val Asp Gly Lys Gln Val Lys Val Gly Gly Val Asp Val				
385		390	395	400

(x) PUBLICATION INFORMATION:

(PATENT NO.: 5,093,240)

(A) AUTHORS: Inouye *et al.*

(C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.

(D) VOLUME: 82

(F) PAGES: 3154-3158

(G) DATE: (1985)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGGGGGGGG GGGGGGGGGG GGGGGGGGGG GGGAAATGCAA TTCATCTTG CATCAAAGAA	60
TTACATCAA TCTCTAGTTG ATCAACTAAA TTGTCTCGAC AACAAACAAGC AAAC ATG	117
Met	
1	
ACA AGC AAA CAA TAC TCA GTC AAG CTT ACA TCA GAC TTC GAC AAC CCA	165
Thr Ser Lys Gln Tyr Ser Val Lys Leu Thr Ser Asp Phe Asp Asn Pro	
5 10 15	
AGA TGG ATT GGA CGA CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC AAC	213
Arg Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val Asn	
20 25 30	
CAC AAT GGA AAA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCA TCT GAT	261
His Asn Gly Lys Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser Asp	
35 40 45	
ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC AAA CGA CAC	309
Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg His	
50 55 60 65	
AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GGA ATG AAA TAT GGT	357
Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Gly Met Lys Tyr Gly	
70 75 80	
TG GAA ACT GAT TGG CCT GCA TAT ATT GAA GGA TGG AAA AAA TTG GCT	405
Val Glu Thr Asp Trp Pro Ala Tyr Ile Glu Gly Trp Lys Lys Leu Ala	
85 90 95	
ACT GAT GAA TTG GAG AAA TAC GCC AAA AAC GAA CCA ACG CTC ATC CGT	453
Thr Asp Glu Leu Glu Lys Tyr Ala Lys Asn Glu Pro Thr Leu Ile Arg	
100 105 110	
ATA TGG GGT GAT GCT TTG TTT GAT ATC GTT GAC AAA GAT CAA AAT GGA	501
Ile Trp Gly Asp Ala Leu Phe Asp Ile Val Asp Lys Asp Gln Asn Gly	
115 120 125	
GCC ATT ACA CTG GAT GAA TGG AAA GCA TAC ACC AAA GCT GCT GGT ATC	549
Ala Ile Thr Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ala Ala Gly Ile	
130 135 140 145	
ATC CAA TCA TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT ATT	597
Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp Ile	
150 155 160	
GAT GAA AGT GGA CAA CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT TTA	645
Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His Leu	
165 170 175	

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His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Gly Met Lys Tyr	
65 70 75 80	
GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC GAA GGA TGG AAA AGA CTG	288
Gly Val Glu Thr Glu Trp Pro Glu Tyr Ile Glu Gly Trp Lys Arg Leu	
85 90 95	
GCT TCC GAG GAA TTG AAA AGG TAT TCA AAA AAC CAA ATC ACA CTT ATT	336
Ala Ser Glu Glu Leu Lys Arg Tyr Ser Lys Asn Gln Ile Thr Leu Ile	
100 105 110	
CGT TTA TGG GGT GAT GCA TTG TTC GAT ATC ATT GAC AAA GAC CAA AAT	384
Arg Leu Trp Gly Asp Ala Leu Phe Asp Ile Ile Asp Lys Asp Gln Asn	
115 120 125	
GGA GCT ATT TCA CTG GAT GAA TGG AAA GCA TAC ACC AAA TCT GAT GGC	432
Gly Ala Ile Ser Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ser Asp Gly	
130 135 140	
ATC ATC CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT	480
Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp	
145 150 155 160	
ATT GAT GAA AGT GGA CAG CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT	528
Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His	
165 170 175	
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT	576
Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly	
180 185 190	
GGA GCT GTC CCC TAA	591
Gly Ala Val Pro *	
195	

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 591 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant Aequorin AEQ2

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Prasher et al.

(B) TITLE: Sequence Comparisons of Complementary
DNAs Encoding Aequorin Isotypes

(C) JOURNAL: Biochemistry

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 591 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant Aequorin AEQ3

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Prasher et al.

(B) TITLE: Sequence Comparisons of Complementary DNAs Encoding Aequorin Isoforms

(C) JOURNAL: Biochemistry

(D) VOLUME: 26

(F) PAGES: 1326-1332

(G) DATE: 1987

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA TCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Ser Asp Phe Asp Asn	
1 5 10 15	
CCA AGA TGG ATT GGA CGA CAC AAG CAT ATG TTC AAT TTC CTT GAT GTC	96
Pro Arg Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AAA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCA TCT	144
Asn His Gly Lys Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTC ATC AAT AAC CTT GGA GCA ACA CCT GAG CAA GCC AAA CGA	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GGA GAC TTC TTC GGA GGA GCT GGA ATG AAA TAT	240
His Lys Asp Ala Val Gly Asp Phe Phe Gly Gly Ala Gly Met Lys Tyr	
65 70 75 80	
GGT GTG GAA ACT GAT TGG CCT GCA TAC ATT GAA GGA TGG AAA AAA TTG	288
Gly Val Glu Thr Asp Trp Pro Ala Tyr Ile Glu Gly Trp Lys Lys Leu	
85 90 95	
GCT ACT GAT GAA TTG GAG AAA TAC GCC AAA AAC GAA CCA ACG CTC ATC	336
Ala Thr Asp Glu Leu Glu Lys Tyr Ala Lys Asn Glu Pro Thr Leu Ile	
100 105 110	
CGT ATA TGG GGT GAT GCT TTG TTC GAT ATC GTT GAC AAA GAT CAA AAT	384
Arg Ile Trp Gly Asp Ala Leu Phe Asp Ile Val Asp Lys Asp Gln Asn	

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20	25	30	
CTT GAC GAG ATG GTC TAC AAG GCG TCC GAT ATT GTT ATA AAC AAT CTT Leu Asp Glu Met Val Tyr Lys Ala Ser Asp Ile Val Ile Asn Asn Leu 35 40 45			144
GGA GCA ACA CCT GAA CAA GCC AAA CGT CAC AAA GAT GCT GTA GAA GCC Gly Ala Thr Pro Glu Gln Ala Lys Arg His Lys Asp Ala Val Glu Ala 50 55 60			192
TTC TTC GGA GGA GCT GCA ATG AAA TAT GGT GTA GAA ACT GAA TGG CCT Phe Phe Gly Gly Ala Ala Met Lys Tyr Gly Val Glu Thr Glu Trp Pro 65 70 75 80			240
GAA TAC ATC GAA GGA TGG AAA AGA CTG GCT TCC GAG GAA TTG AAA AGG Glu Tyr Ile Glu Gly Trp Lys Arg Leu Ala Ser Glu Glu Leu Lys Arg 85 90 95			288
TAT TCA AAA AAC CAA ATC ACA CTT ATT CGT TTA TGG GGT GAT GCA TTG Tyr Ser Lys Asn Gln Ile Thr Leu Ile Arg Leu Trp Gly Asp Ala Leu 100 105 110			336
TTC GAT ATC ATT GAC AAA GAC CAA AAT GGA GCT ATT TCA CTG GAT GAA Phe Asp Ile Ile Asp Lys Asp Gln Asn Gly Ala Ile Ser Leu Asp Glu 115 120 125			384
TGG AAA GCA TAC ACC AAA TCT GCT GGC ATC ATC CAA TCG TCA GAA GAT Trp Lys Ala Tyr Thr Lys Ser Ala Gly Ile Ile Gln Ser Ser Glu Asp 130 135 140			432
TGC GAG GAA ACA TTC AGA GTG TGC GAT ATT GAT GAA AGT GGA CAG CTC Cys Glu Glu Thr Phe Arg Val Cys Asp Ile Asp Glu Ser Gly Gln Leu 145 150 155 160			480
GAT GTT GAT GAG ATG ACA AGA CAA CAT TTA GGA TTT TGG TAC ACC ATG Asp Val Asp Glu Met Thr Arg Gln His Leu Gly Phe Trp Tyr Thr Met 165 170 175			528
GAT CCT GCT TGC GAA AAG CTC TAC GGT GGA GCT GTC CCC Asp Pro Ala Cys Glu Lys Leu Tyr Gly Gly Ala Val Pro 180 185			567

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence

- (B) LOCATION: 1...588

- (D) OTHER INFORMATION: Aequorin mutant w/increased bioluminescence activity

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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...588

(D) OTHER INFORMATION: Recombinant site-directed Aequorin mutant
w/increased biolum. activity

(x) PUBLICATION INFORMATION:

PATENT NO.: 5,360,728

(K) RELEVANT RESIDUES IN SEQ ID NO: 11:

Glu 135 changed to Ser

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATG ACC AGC GAA CAA TAC TCA GTC AAG CTT ACA CCA GAC TTC GAC AAC	48
Met Thr Ser Glu Gln Tyr Ser Val Lys Leu Thr Pro Asp Phe Asp Asn	
1 5 10 15	
CCA AAA TGG ATT GGA CGA CAC AAG CAC ATG TTT AAT TTT CTT GAT GTC	96
Pro Lys Trp Ile Gly Arg His Lys His Met Phe Asn Phe Leu Asp Val	
20 25 30	
AAC CAC AAT GGA AGG ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCC	144
Asn His Asn Gly Arg Ile Ser Leu Asp Glu Met Val Tyr Lys Ala Ser	
35 40 45	
GAT ATT GTT ATA AAC AAT CTT GGA GCA ACA CCT GAA CAA GCC AAA CGT	192
Asp Ile Val Ile Asn Asn Leu Gly Ala Thr Pro Glu Gln Ala Lys Arg	
50 55 60	
CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GCA ATG AAA TAT	240
His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Ala Met Lys Tyr	
65 70 75 80	
GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC GAA GGA TGG AAA AGA CTG	288
Gly Val Glu Thr Glu Trp Pro Glu Tyr Ile Glu Gly Trp Lys Arg Leu	
85 90 95	
GCT TCC GAG GAA TTG AAA AGG TAT TCA AAA AAC CAA ATC ACA CTT ATT	336
Ala Ser Glu Glu Leu Lys Arg Tyr Ser Lys Asn Gln Ile Thr Leu Ile	
100 105 110	
CGT TTA TGG GGT GAT GCA TTG TTC GAT ATC ATT TCC AAA GAC CAA AAT	384
Arg Leu Trp Gly Asp Ala Leu Phe Asp Ile Ile Ser Lys Asp Gln Asn	
115 120 125	

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CAC AAA GAT GCT GTA GAA GCC TTC TTC GGA GGA GCT GCA ATG AAA TAT His Lys Asp Ala Val Glu Ala Phe Phe Gly Gly Ala Ala Met Lys Tyr 65 70 75 80	240
GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC GAA GGA TGG AAA AGA CTG Gly Val Glu Thr Glu Trp Pro Glu Tyr Ile Glu Gly Trp Lys Arg Leu 85 90 95	288
GCT TCC GAG GAA TTG AAA AGG TAT TCA AAA AAC CAA ATC ACA CTT ATT Ala Ser Glu Glu Leu Lys Arg Tyr Ser Lys Asn Gln Ile Thr Leu Ile 100 105 110	336
CGT TTA TGG GGT GAT GCA TTG TTC GAT ATC ATT TCC AAA GAC CAA AAT Arg Leu Trp Gly Asp Ala Leu Phe Asp Ile Ile Ser Lys Asp Gln Asn 115 120 125	384
GCA GCT ATT TCA CTG GAT GAA TGG AAA GCA TAC ACC AAA TCT GCT GGC Ala Ala Ile Ser Leu Asp Glu Trp Lys Ala Tyr Thr Lys Ser Ala Gly 130 135 140	432
ATC ATC CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT Ile Ile Gln Ser Ser Glu Asp Cys Glu Glu Thr Phe Arg Val Cys Asp 145 150 155 160	480
ATT GAT GAA AGT GGA CAG CTC GAT GTT GAT GAG ATG ACA AGA CAA CAT Ile Asp Glu Ser Gly Gln Leu Asp Val Asp Glu Met Thr Arg Gln His 165 170 175	528
TTA GGA TTT TGG TAC ACC ATG GAT CCT GCT TGC GAA AAG CTC TAC GGT Leu Gly Phe Trp Tyr Thr Met Asp Pro Ala Cys Glu Lys Leu Tyr Gly 180 185 190	576
GGA GCT GTC CCC Gly Ala Val Pro 195	588

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 567 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...567
- (D) OTHER INFORMATION: Recombinant apoaequorin (AQUALITE[®])

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTC AAG CTT ACA CCA GAC TTC GAC AAC CCA AAA TGG ATT GGA CGA CAC Val Lys Leu Thr Pro Asp Phe Asp Asn Pro Lys Trp Ile Gly Arg His 1 5 10 15	48
AAG CAC ATG TTT AAT TTT CTT GAT GTC AAC CAC AAT GGA AGG ATC TCT Lys His Met Phe Asn Phe Leu Asp Val Asn His Asn Gly Arg Ile Ser 20 25 30	96

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20	25	30
Gly Gln Tyr Leu Thr Val Val Met	Gly Glu Lys Asp	Lys Arg Pro Phe
35	40	45
Ser Ile Ala Ser Ser Pro Cys Arg His	Glu Gly Glu Ile Glu Leu His	
50	55	60
Ile Gly Ala Ala Glu His Asn Ala Tyr Ala Gly Glu Val Val Glu Ser		
65	70	75
Met Lys Ser Ala Leu Glu Thr Gly Gly Asp Ile Leu Ile Asp Ala Pro		
85	90	95
His Gly Glu Ala Trp Ile Arg Glu Asp Ser Asp Arg Ser Met Leu Leu		
100	105	110
Ile Ala Gly Gly Thr Gly Phe Ser Tyr Val Arg Ser Ile Leu Asp His		
115	120	125
Cys Ile Ser Gln Gln Ile Gln Lys Pro Ile Tyr Leu Tyr Trp Gly Gly		
130	135	140
Arg Asp Glu Cys Gln Leu Tyr Ala Lys Ala Glu Leu Glu Ser Ile Ala		
145	150	155
160		
Gln Ala His Ser His Ile Thr Phe Val Pro Val Val Glu Lys Ser Glu		
165	170	175
Gly Trp Thr Gly Lys Thr Gly Asn Val Leu Glu Ala Val Lys Ala Asp		
180	185	190
Phe Asn Ser Leu Ala Asp Met Asp Ile Tyr Ile Ala Gly Arg Phe Glu		
195	200	205
Met Ala Gly Ala Ala Arg Glu Gln Phe Thr Thr Glu Lys Gln Ala Lys		
210	215	220
Lys Glu Gln Leu Phe Gly Asp Ala Phe Ala Phe Ile		
225	230	235

7. The device of claim 5, wherein the luciferase is a photoprotein.

8. The device of claim 5, wherein the bioluminescence generating system is selected from the group consisting of the

5 *Aequorea, Vargula, Renilla, Obelin, Porichthys, Odontosyllis, Aristostomias, Pachystomias*, firefly, and bacterial systems.

9. The microelectronic device of claim 1, wherein the substrate is a semiconductor substrate comprising a surface that is adapted for linking macromolecules, each micro-location being defined
10 by a portion of the surface that is adapted to allow the separate chemical reactant at that micro-location to be coupled thereto.

10. The device of claim 9, wherein the surface is coated with an inert material that is derivatized for linking macromolecules.

11. The microelectronic device of claim 1, wherein the substrate is a semiconductor substrate comprising a surface, each micro-location being defined by a portion of the surface, and each photodetector includes a photodiode located at the portion of the surface at the respective micro-location, the photodiode converting photons of light emitted by the chemical reaction at that micro-location
20 into a photocurrent that defines the sensed signal.

12. The microelectronic device of claim 11, wherein the electronic circuit includes a pixel unit cell circuit associated with each photodiode and a delta-sigma A/D conversion circuit, each pixel unit cell circuit being configured to integrate the sensed signal from the
25 respective photodiode and the A/D conversion circuit being configured to quantize the integrated sensed signals.

13. The microelectronic device of claim 12, wherein each pixel unit cell circuit is addressable and the electronic circuit further includes an address control circuit for sequentially addressing each pixel unit cell
30 circuit, and wherein the A/D conversion circuit quantizes the integrated

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19. A microelectronic device of claim 1, comprising:
 - a substrate;
 - micro-locations defined on the substrate that are for receiving a fluid sample for analysis, each micro-location comprising an attachment layer to which macromolecules are linked;
 - a macromolecule linked to a plurality of the micro-locations via the attachment layer, wherein the macromolecule selectively binds to analyte present in the sample received by the device;
 - an independent photodetector optically coupled to each micro-location, wherein each photodetector is configured to generate a sensed signal responsive to photons of light emitted at the corresponding micro-location when the selected analyte bound at that micro-location is exposed to a second macromolecule that binds to the first macromolecule or analyte linked to one or more components of a light-emitting reaction in the presence of the remaining components of the light-emitting reaction; and
 - an electronic circuit coupled to each photodetector and configured to read the sensed signal generated by each photodetector and to generate output data signals therefrom that are indicative of the light emitted at each micro-location by the light-emitting reaction, wherein the device detects or identifies analytes in a fluid sample using light-emitting reactions.
20. The device of claim 19, wherein each macromolecule is an antibody and the analyte is an antigen.
21. The device of claim 19, wherein the an array of micro-locations defined on the substrate for receiving the fluid sample to be analyzed form wells in the surface of the device.
22. The device of claim 21, wherein one or a plurality of the wells comprise a reflective material disposed along the sides thereof or

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33. The microelectronic device of claim 19, wherein the device comprises a plurality of different macromolecules each specific for a different analyte, each different macromolecule present at a different micro-location.

5 34. The microelectronic device of claim 19, wherein:
the micro-locations are in the form of an array;
the array of micro-locations includes a first and a second array of pixel elements comprising a first and a second size, respectively, the first and second sizes being different.

10 35. The microelectronic device of claim 34, wherein the receptor antibody attached to the attachment layer of the first pixel element array is specific to bind a first selected analyte and the receptor antibody attached to the attachment layer of the second pixel element array is specific to bind a second selected analyte different than the first

15 selected analyte.

36. The microelectronic device of claim 19, wherein each micro-location is located on a surface of a semiconductor substrate, with the surface at each micro-location defining the attachment layer for that micro-location.

20 37. The microelectronic device of claim 36, wherein the surface of the semiconductor substrate is derivatized to enhance the attachment of the receptor antibody to the attachment layer at each micro-location.

25 38. The microelectronic device of claim 37, wherein each photodetector includes a photodiode located at the surface of the respective micro-location, and the reaction produces photons of light converted by the photodiode into a photocurrent when the selected analyte is present in the sample, the photocurrent being the sensed signal generated by the photodiode.

30 39. The microelectronic device of claim 38, wherein the electronic circuit includes a pixel unit cell circuit associated with each

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correlated with each micro-location being indicative of the bioluminescence emitted at that micro-location.

44. The device of any of claims 1-42, wherein the micro-locations are provided as an array.

5 45. A method of detecting and identifying analytes in a biological sample, comprising the steps of:

providing the microelectronic device of any of claims 1-43;

attaching a macromolecule or plurality of different macromolecules to the surface at each micro-location on the device, wherein

10 macromolecule is specific for binding to selected analyte that may be present in the biological sample;

contacting the sample with the surface of the microelectronic device, whereby any of the selected analytes that are present in the sample bind to the macromolecule attached to the surface at each

15 micro-location;

exposing the surface of the microelectronic device to a second macromolecule or plurality thereof bind to the selected analyte already bound to the first macromolecule at each micro-location, wherein the second macromolecule comprises a component of a bioluminescence

20 generating reaction;

initiating the bioluminescence generating reaction by contacting the surface of the device with the remaining components of the bioluminescence generating reaction;

detecting photons of light emitted by the bio-luminescent reaction

25 using a photodetector optically coupled to each micro-location, each photodetector generating a sensed signal representative of the bioluminescence generation at the respective micro-location.

46. The method of claim 45, further comprising reading the sensed signal generated by each photodetector and generating output

configured to read the output data signals received by the input interface circuit, to correlate the output data signals with the corresponding micro-locations, to integrate the output data signals correlated with each micro-location for a desired time period by accumulating the output data signals in the data acquisition array, and to generate the output device signal which, when applied to the output device, causes the output device to generate visible indicia related to the presence of the selected analytes in the sample.

52. The system of claim 51, wherein the microelectronic device
10 comprises:

an array of micro-locations for receiving the biological sample to be analyzed, each micro-location comprising an attachment layer;

a separate antibody attached to the attachment layer of each micro-location, each antibody specific for binding a selected analyte

15 present in the sample received by the array;

a photodetector optically coupled to each micro-location, each photodetector being configured to generate a sensed signal responsive to bioluminescence emitted at the corresponding micro-location; and

an electronic circuit coupled to each photodetector and configured

20 to read the sensed signal generated by each photodetector and generate output data signals therefrom that are indicative of the bioluminescence emitted at each micro-location by the luciferase-luciferin reaction

53. The system of claim 52, wherein:

each micro-location is located on a surface of a semiconductor

25 substrate and each photodetector includes a photodiode located at the surface at the respective micro-location,

the bioluminescence generating reaction producing photons of light that are converted by the photodiode into a photocurrent when the selected analyte is present; and

30 the photocurrent is a sensed signal generated by the photodiode.

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selected from the group consisting of *Aequorea*, *Vargula*, *Renilla*, *Obelin*, *Porichthys*, *Odontosyllis*, *Aristostomias*, *Pachystomias*, firefly, and bacterial systems.

61. A kit comprising a diagnostic system for detecting

5 infectious agents, comprising:

(a) the microelectronic device of any of claims 1-43;

(b) an anti-ligand;

10 (c) a first composition comprising a conjugate that comprises a component of a bioluminescence generating system, and an anti ligand, wherein the anti ligand specifically binds to an epitope on the surface of the infectious agent; and

(d) a second composition, comprising another component of the bioluminescence generating system.

62. The kit of claim 61, wherein the component of the

15 bioluminescence generating system is a luciferase or luciferin.

63. The kit of claim 61, wherein:

the compositions comprise a bioluminescence generating system;

the bioluminescence generating system comprises a luciferase and a luciferin.

20 64. The kit of claim 61, wherein the bioluminescence generating system is selected from the group consisting of those isolated from the ctenophores, coelenterases, mollusca, fish, ostracods, insects, bacteria, a crustacea, annelids, and earthworms.

25 65. The kit of claim 62, wherein the luciferase is selected from the group consisting of *Aequorea*, *Vargula*, *Renilla*, *Obelin*, *Porichthys*, *Odontosyllis*, *Aristostomias*, *Pachystomias*, firefly, and bacterial systems.

66. The kit of claim 61, further comprising a composition comprising a fluorescent protein.

a fluid dispensing means in association with the base for delivering fluid to the surface of the derivatized silicon substrate;

a first electronic circuit coupled to the photodetector and configured to read the sensed signal generated by each photodetector
5 and to generate output data signals;

a computer processor operably associated with the electronic circuit for receiving and processing the output data signals;

a second electronic circuit in operable association with the computer processor for receiving electronic signals for linking to a
10 muscle or muscle fiber of an animal, wherein the muscle or muscle fiber controls extensor motor control; and

a third electronic circuit in operable association with the computer processor for receiving electronic signals for linking to a muscle or muscle fiber of an animal, wherein the muscle or muscle fiber controls
15 flexor motor control.

73. A method of bypassing spinal cord lesions in an animal using a synthetic neuronal synapse of claim, comprising

drilling microholes into the spinal cord of an animal at predetermined stereotaxic locations flanking a spinal cord lesion;

20 implanting the microelectronic device of claim 72 into the spinal cord at the predetermined stereotaxic location in operable association with a neuron or bundle of neurons;

adding neuronal growth factors through a the fluid dispensing means of the artificial synapse to promote neuronal outgrowth to
25 produce a silica surface neuronal interface; and

implanting the second and third electronic circuits in a predetermined muscle or muscle fiber in a preselected limb of the animal distal to the spinal cord region,

30 whereby upon neurotransmission from the neuron or nerve fiber of the spinal cord muscle movement is effected.

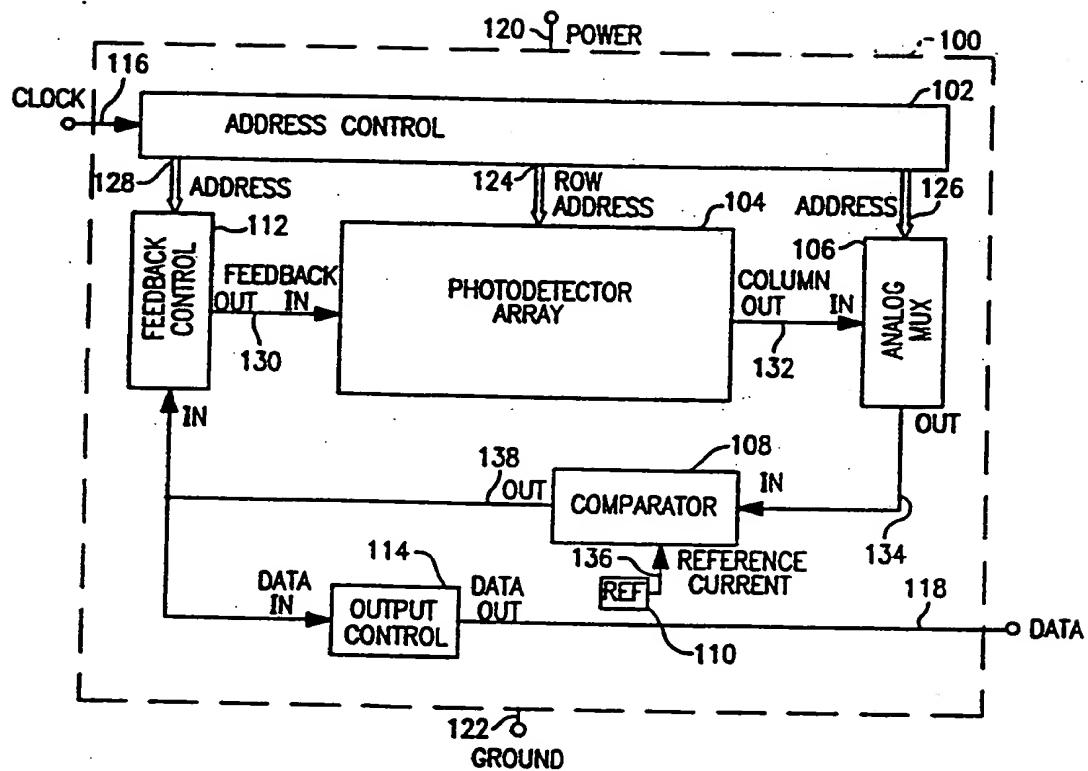
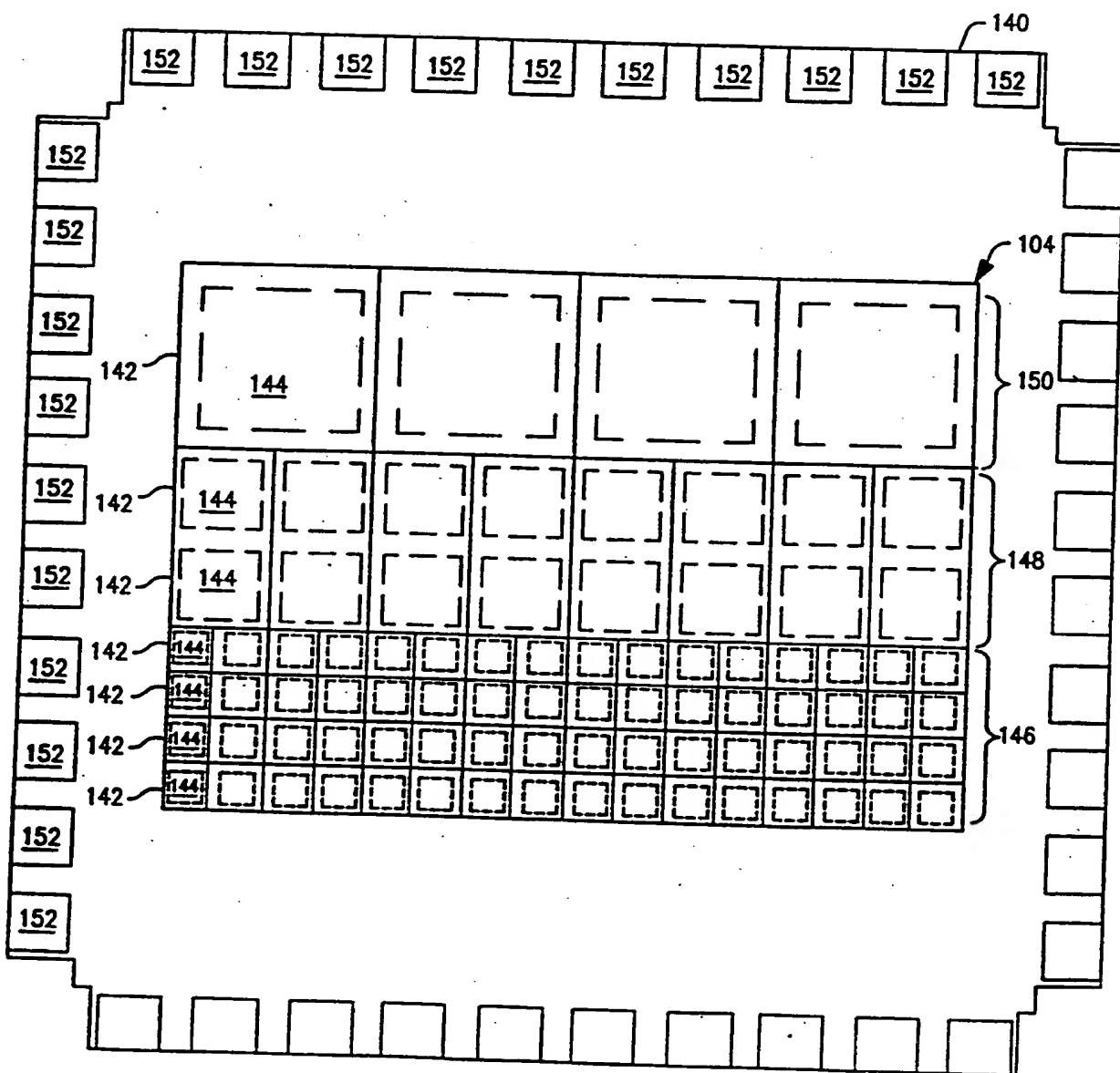


FIG. I

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**FIG. 2**

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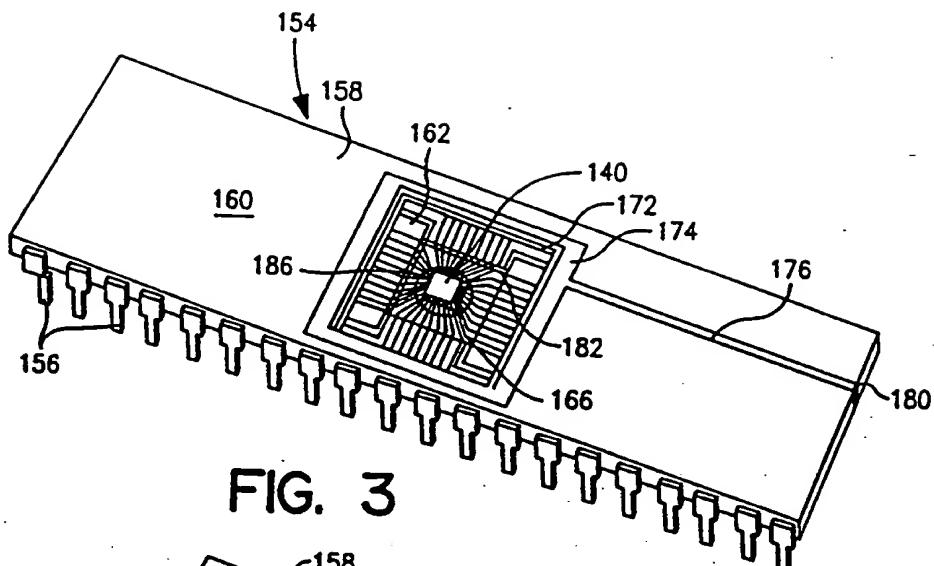


FIG. 3

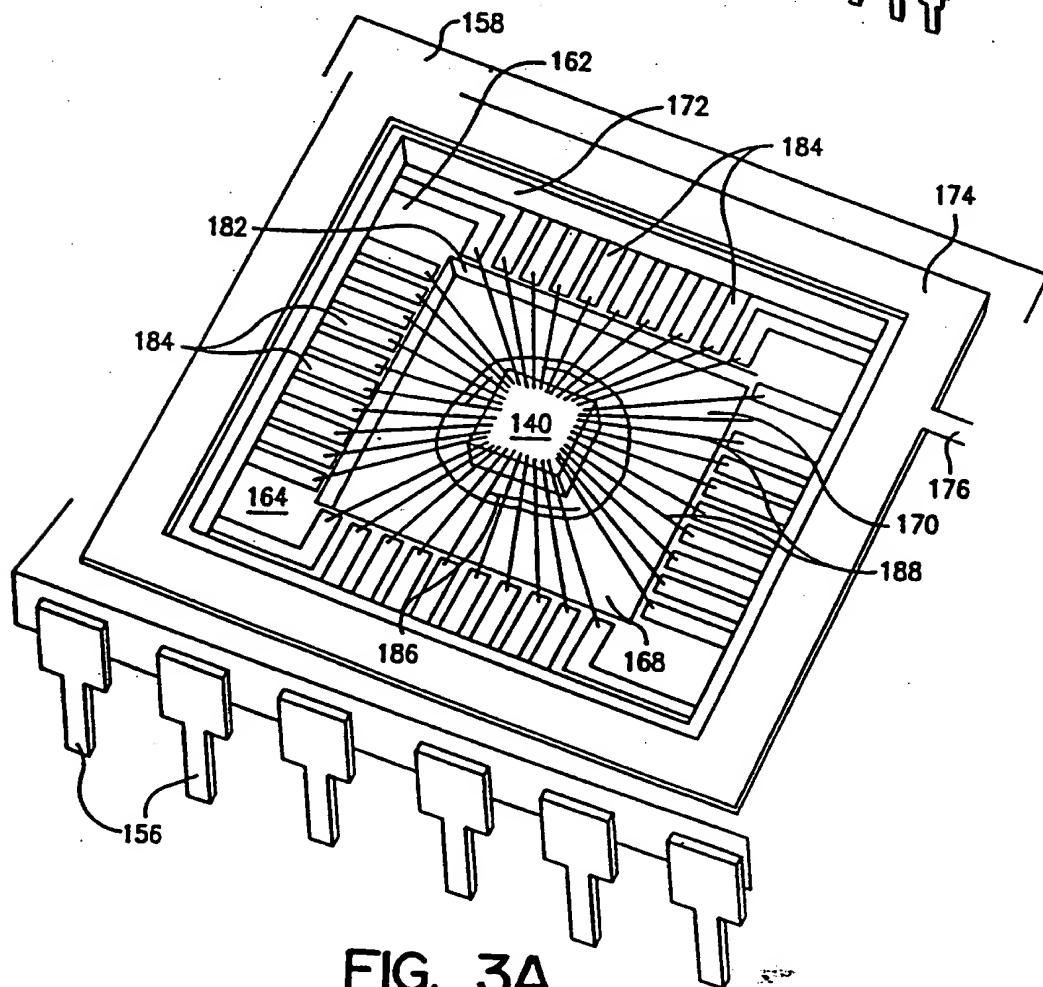


FIG. 3A

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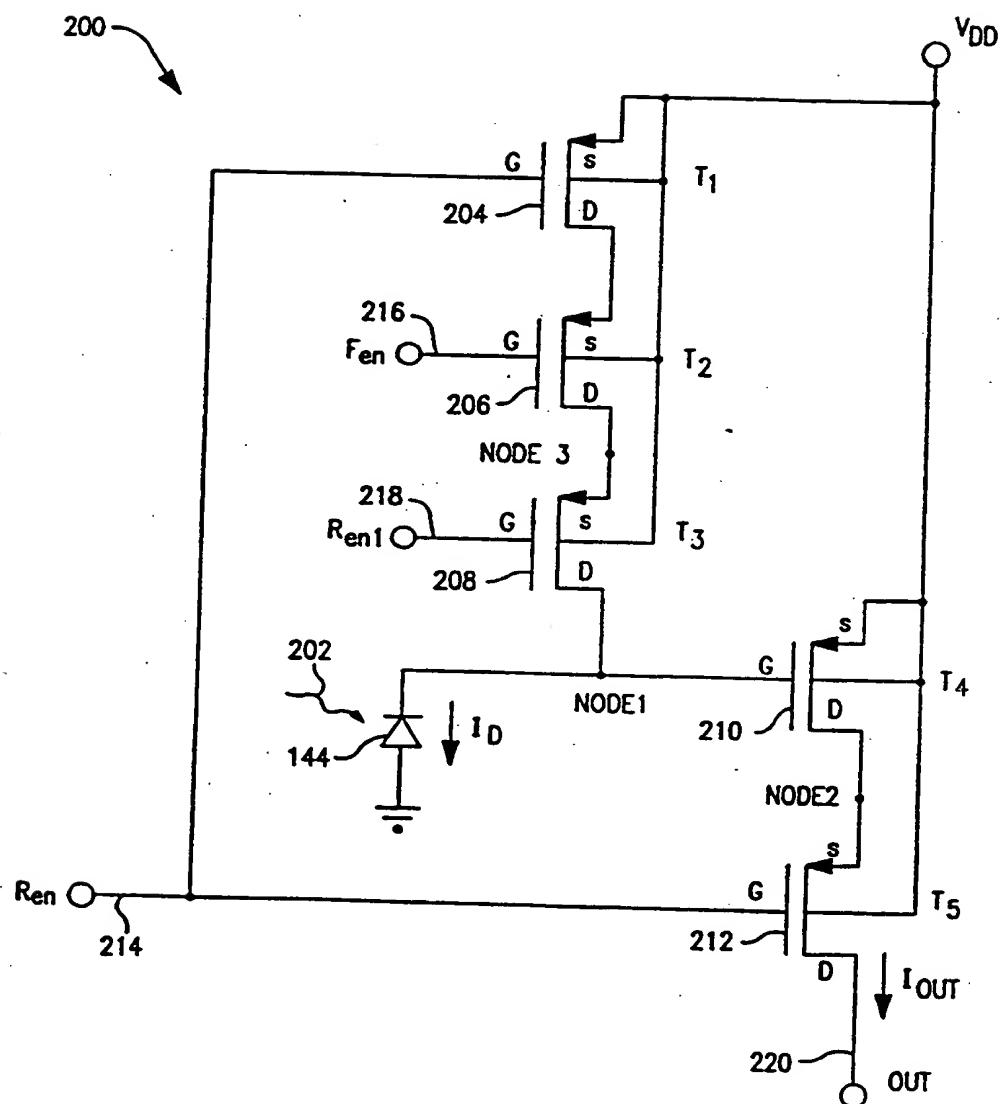


FIG. 4

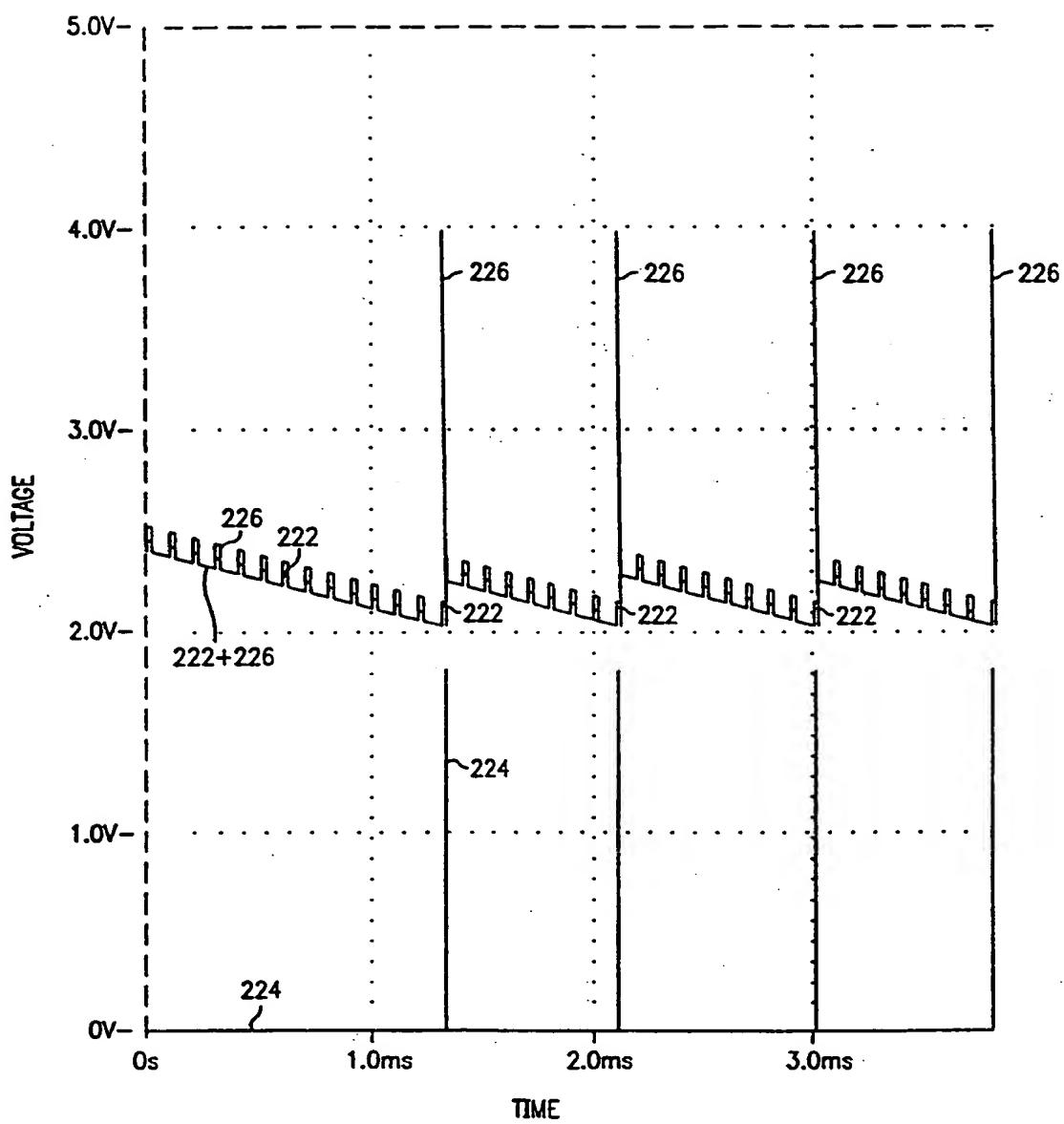


FIG. 5

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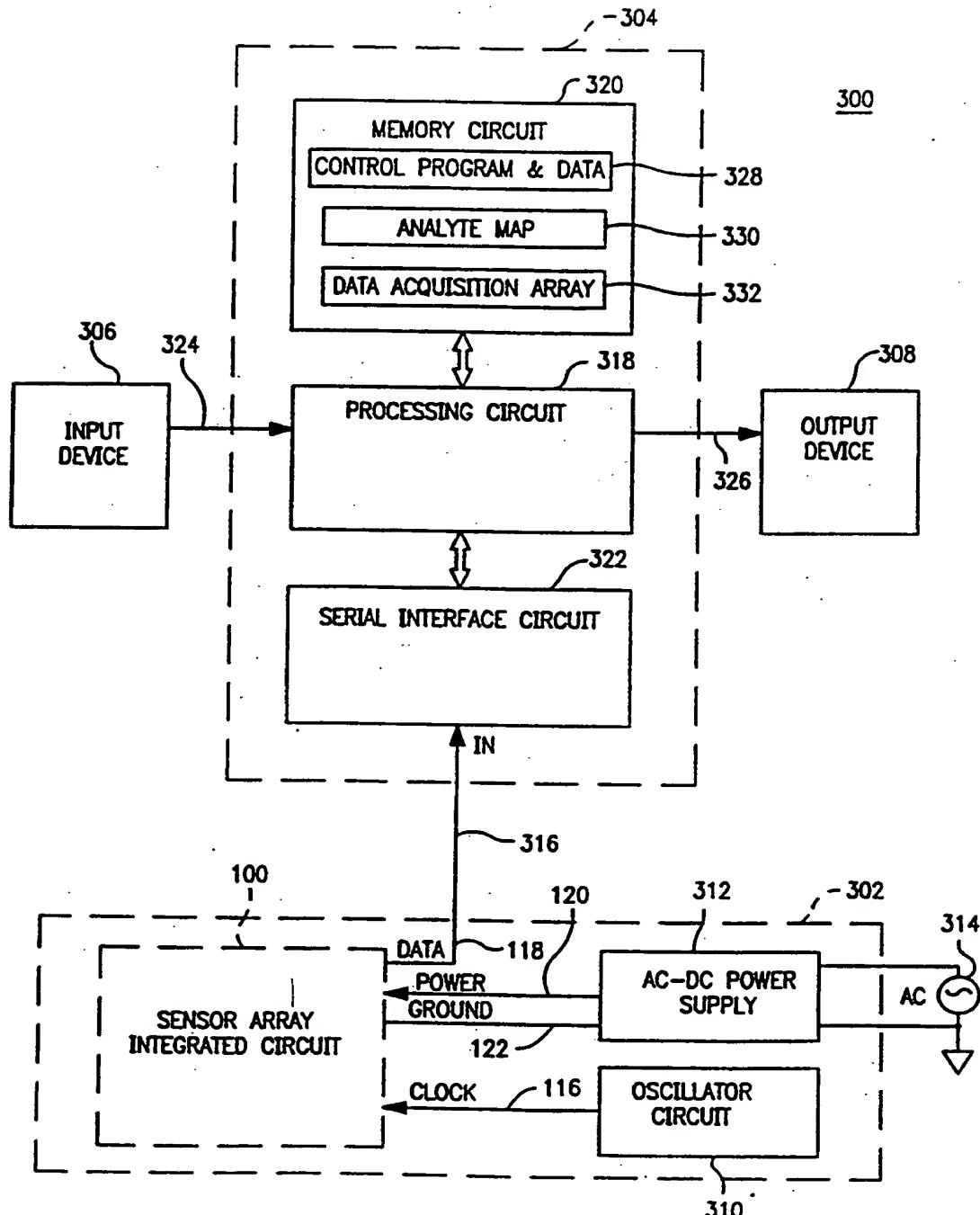


FIG. 6

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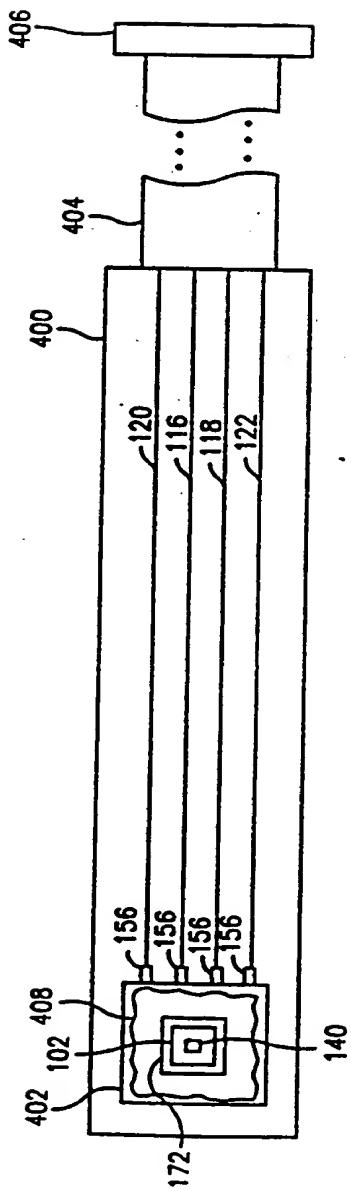


FIG. 7

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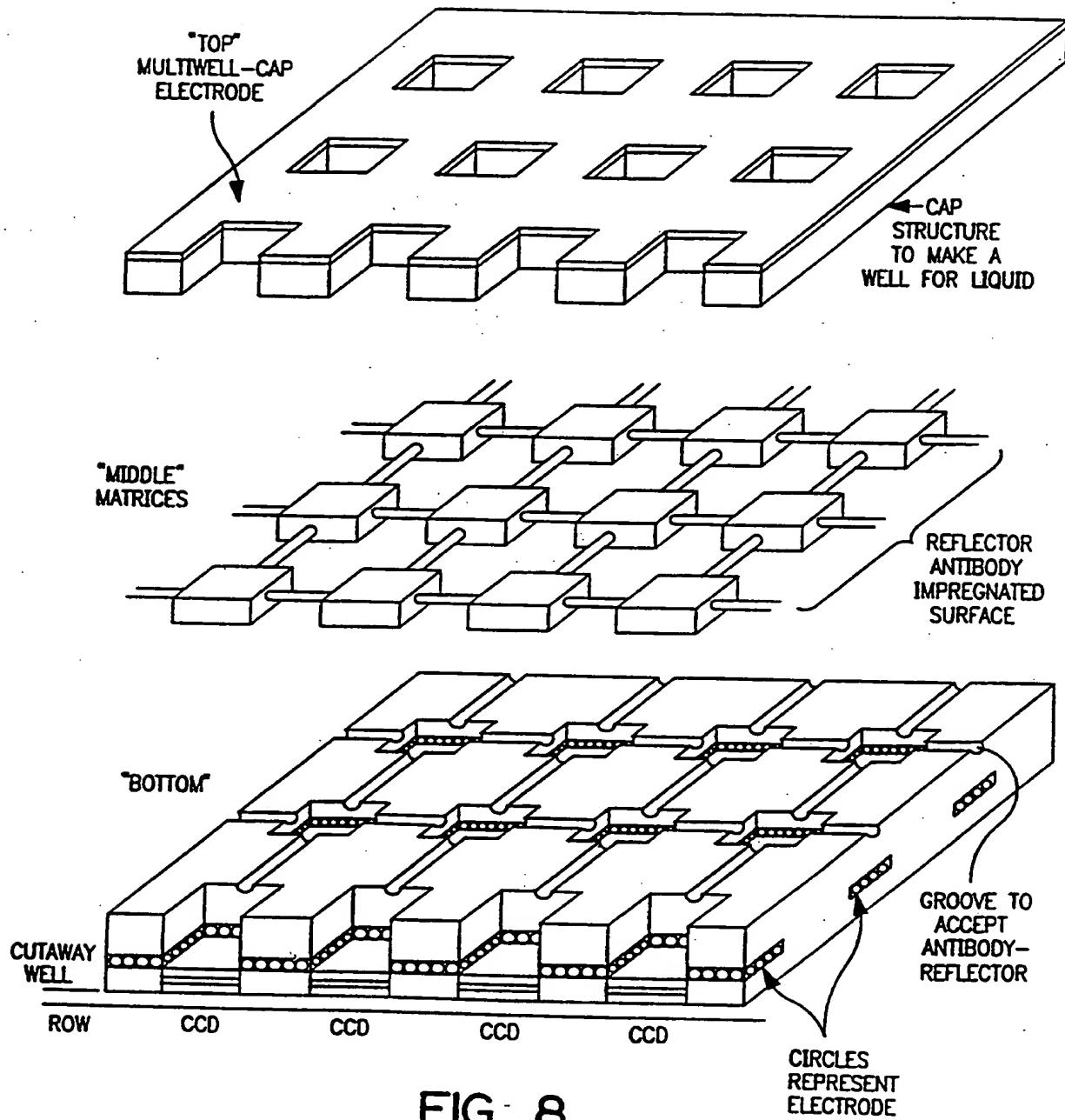


FIG. 8

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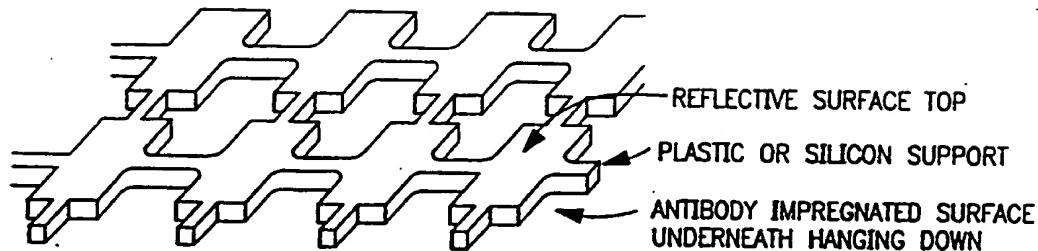


FIG. 9A

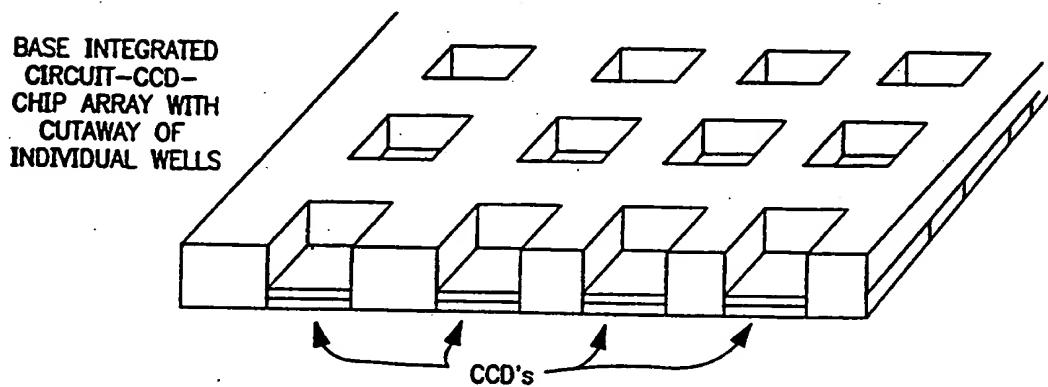


FIG. 9B

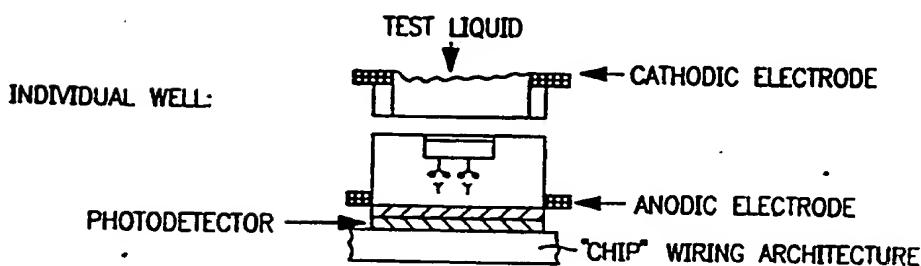


FIG. 9C

SUBSTITUTE SHEET (RULE 26)

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COMPONENTS OF CHIP:

A: CCD SILICON CHARGE COUPLE DEVICE (OTHER CCD'S CAN BE USED DEPENDENT UPON EMITTED LIGHT WAVELENGTH FROM LUCIFERASE)

REFLECTIVE ANTIBODY SURFACE/REACTION SURFACE
AN EXAMPLE WOULD BE A HEAT STABLE MYLAR REFLECTOR

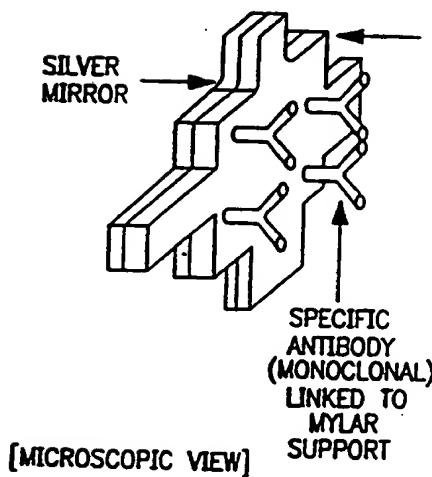


FIG. 10A

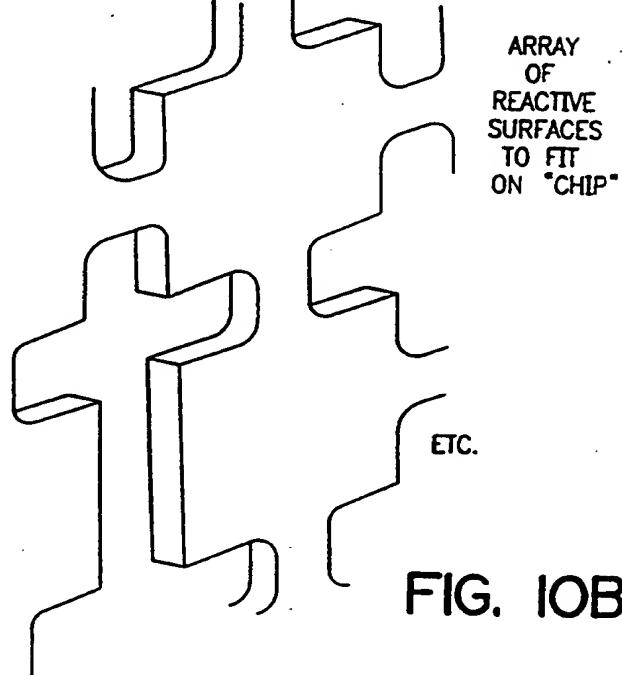


FIG. 10B

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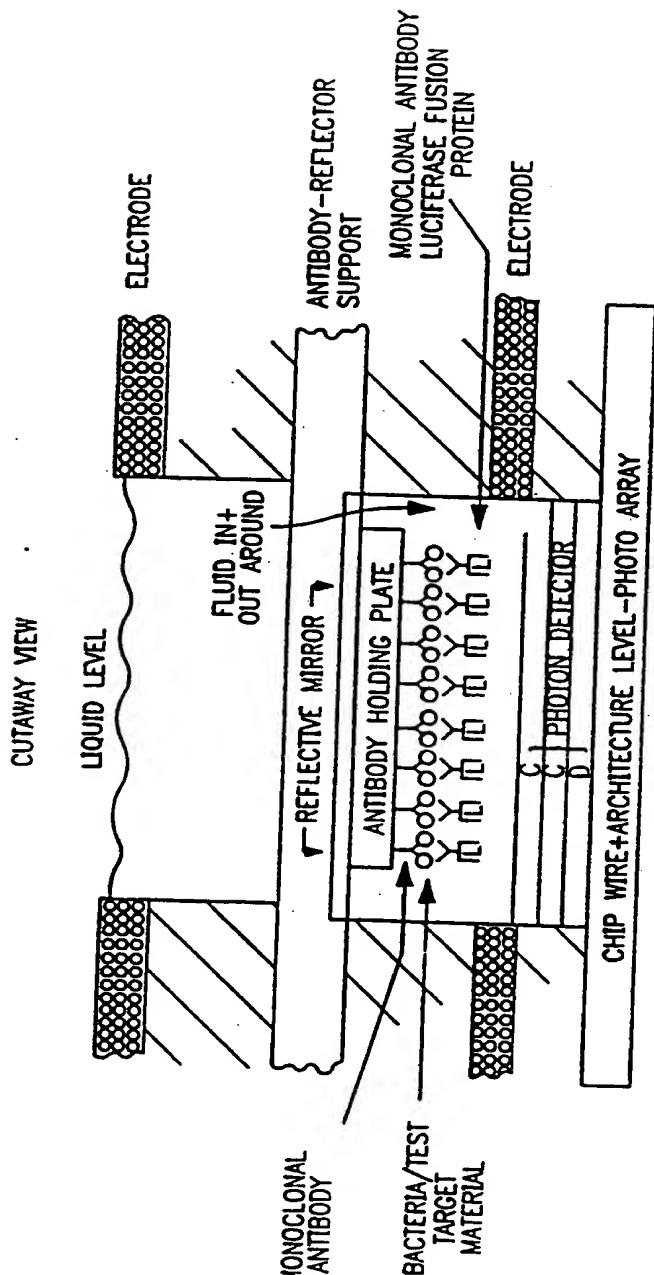
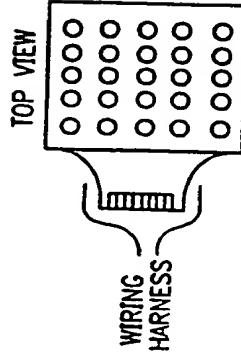


FIG. II



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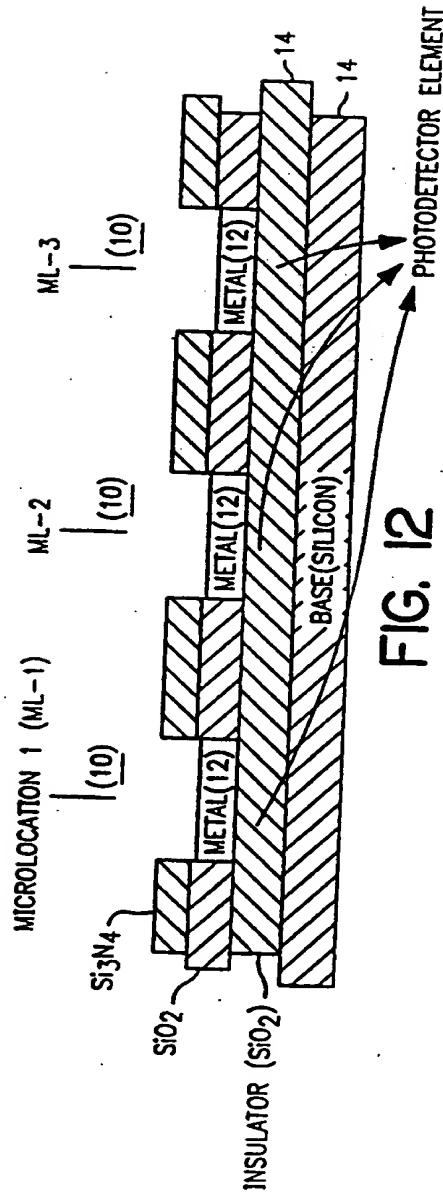


FIG. 12

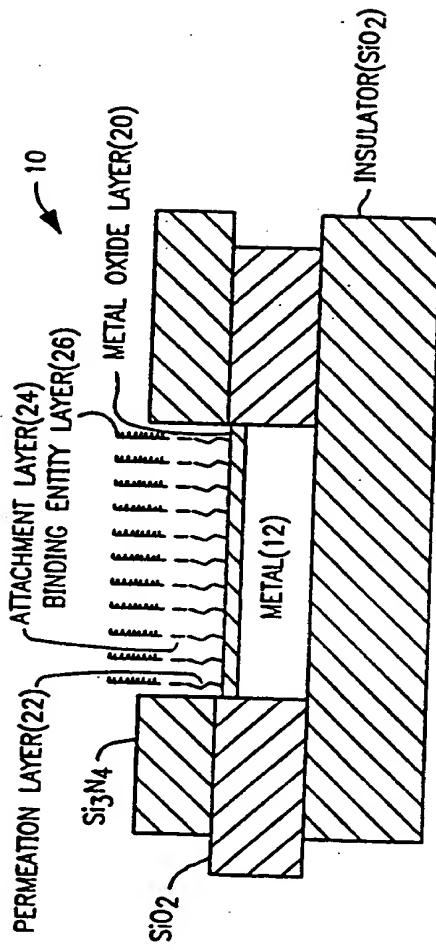


FIG. 13

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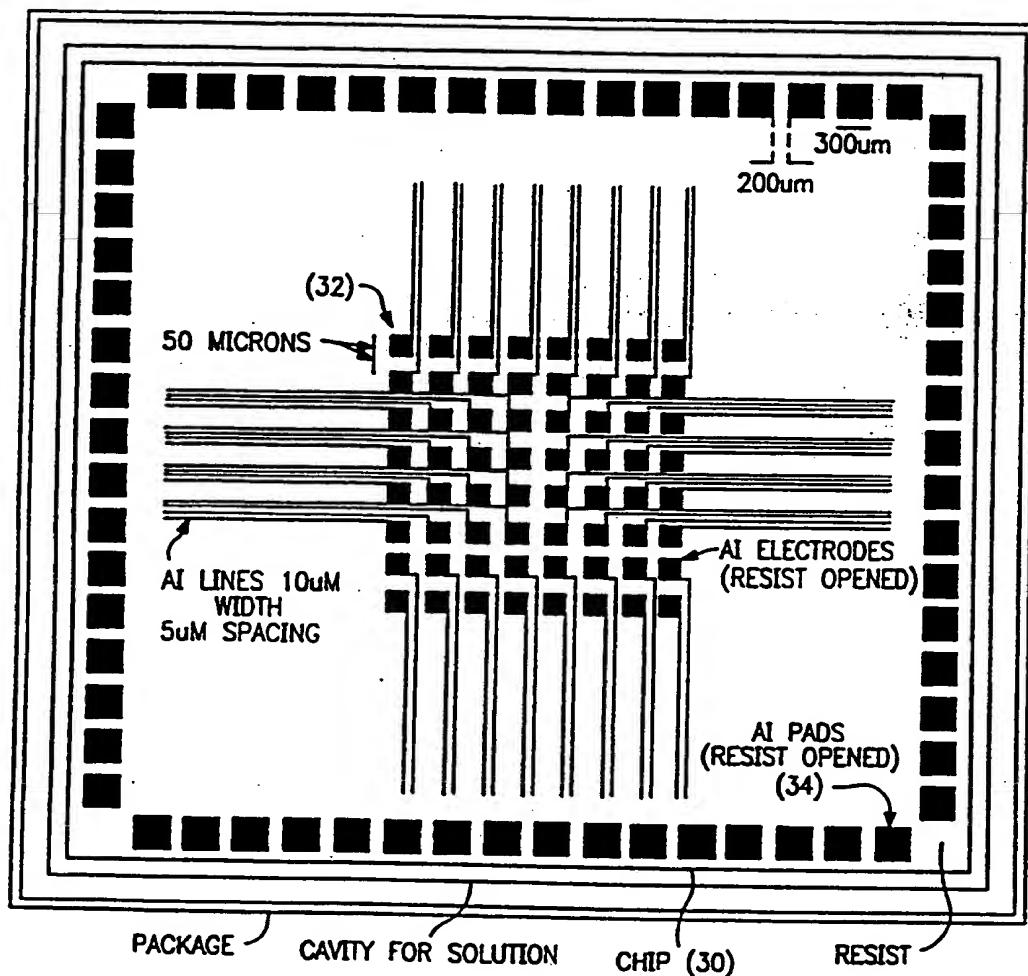
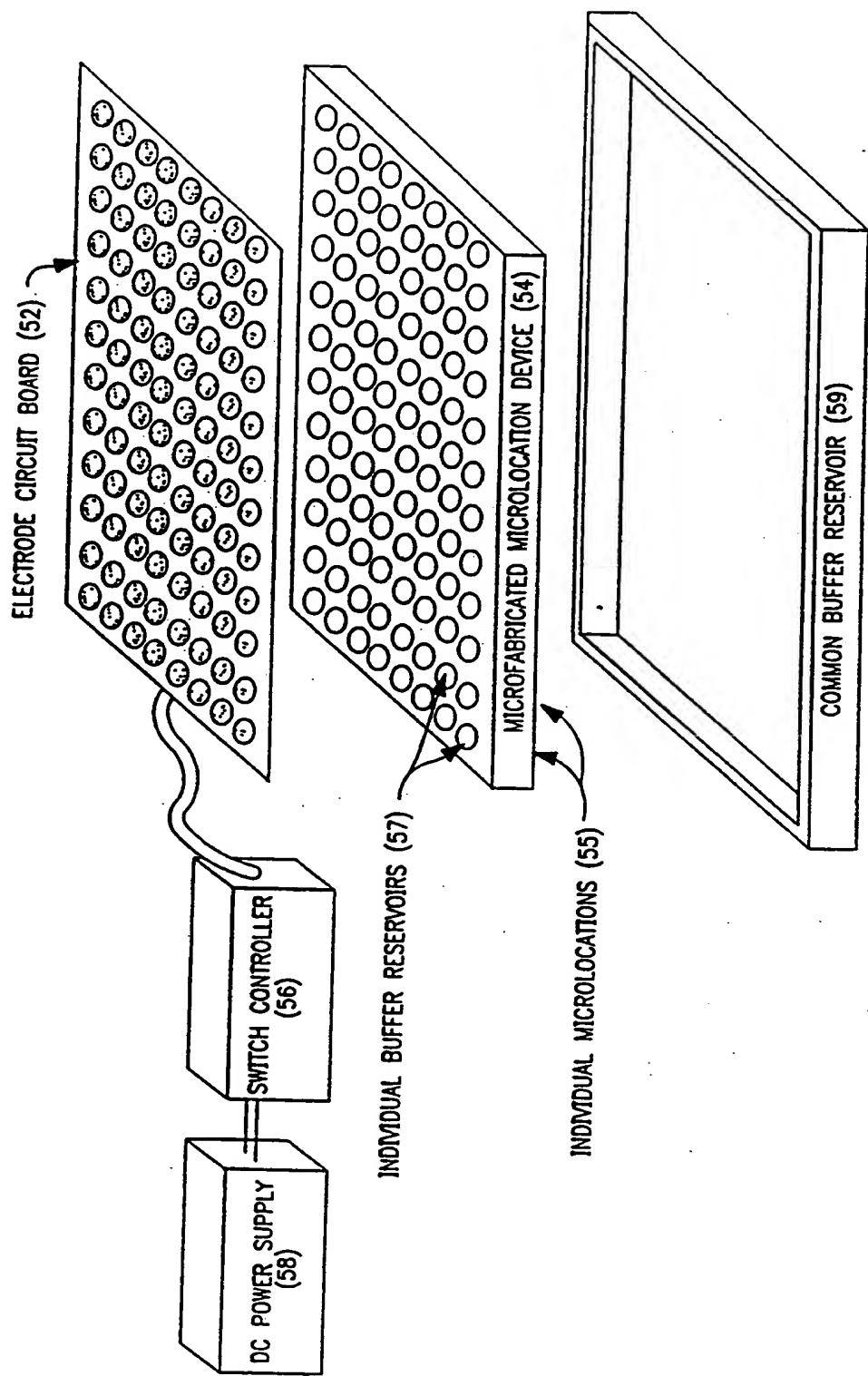


FIG. 14

**FIG. 15**

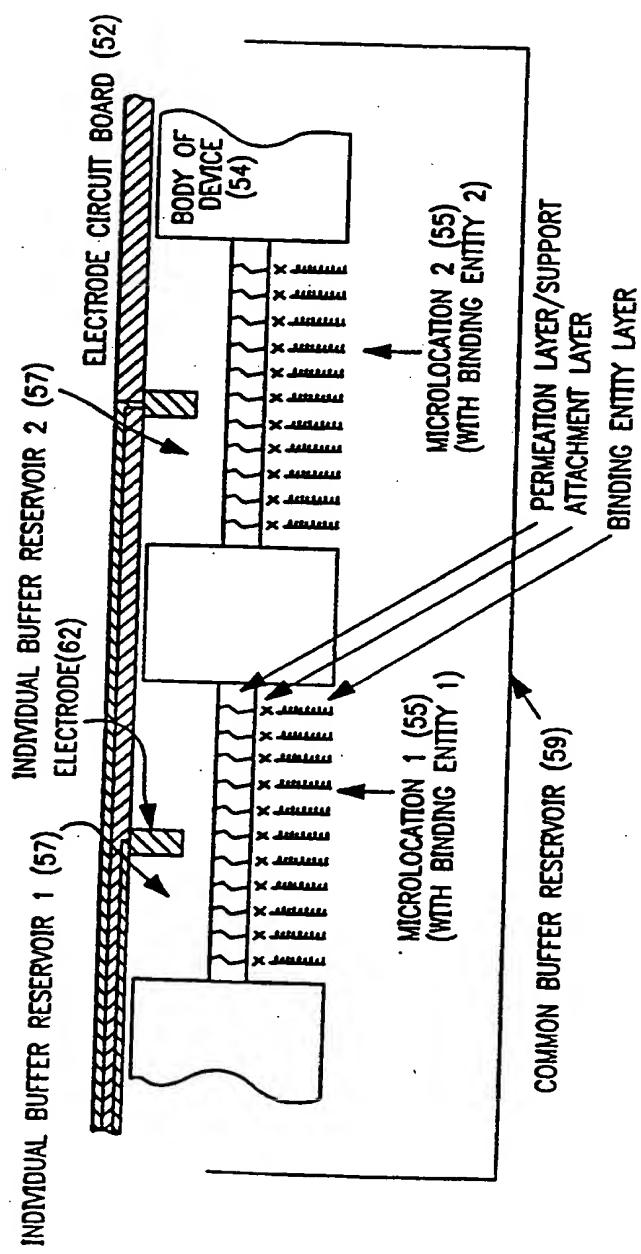


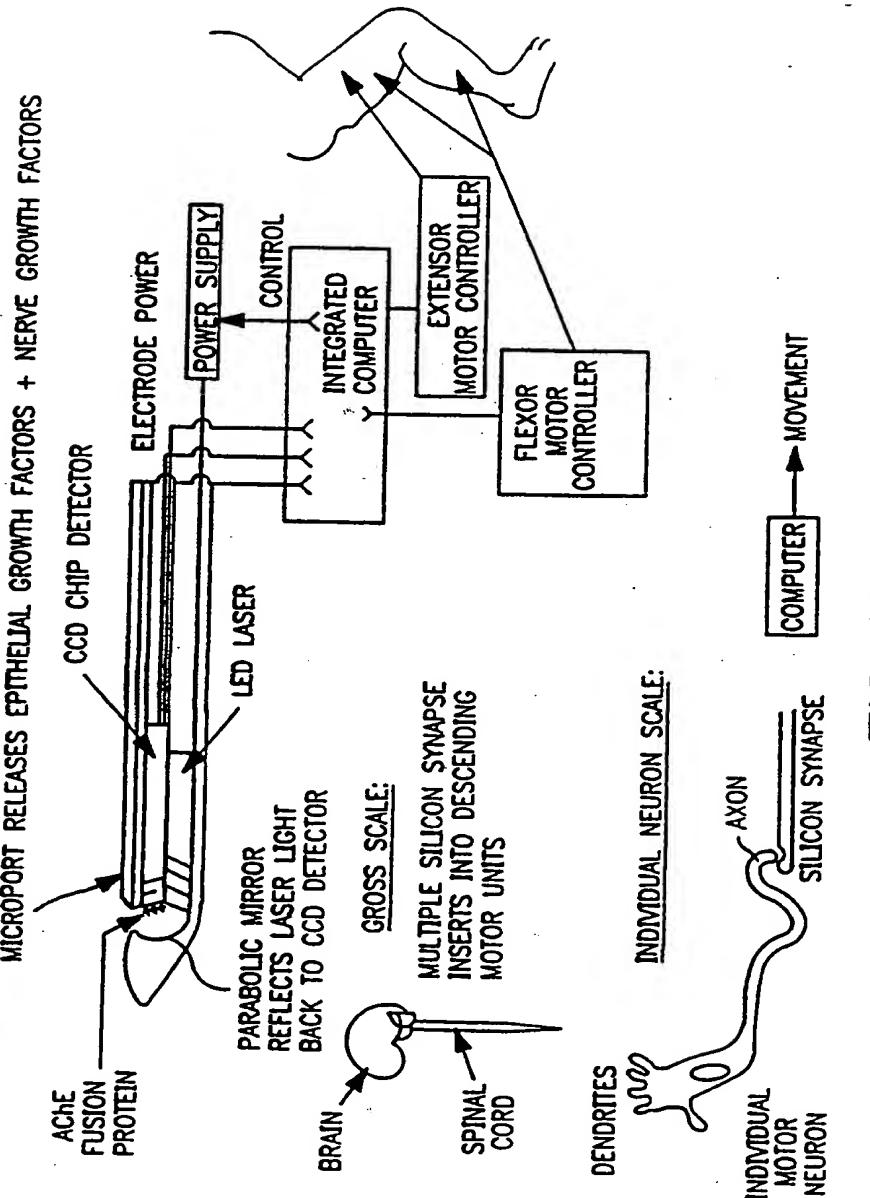
FIG. 16

SILICON-SYNAPE

PURPOSE: TO PROVIDE NEURONAL INPUT TO INTERFACE WITH A COMPUTER FOR THE PURPOSE OF BYPASSING SPINAL CORD LESIONS SO LIMITED MOTOR CONTROL CAN BE BROUGHT TO MUSCLES DISTAL TO THE SPINAL LESION

BLOW-UP DIAGRAM: MOLECULAR SCALE

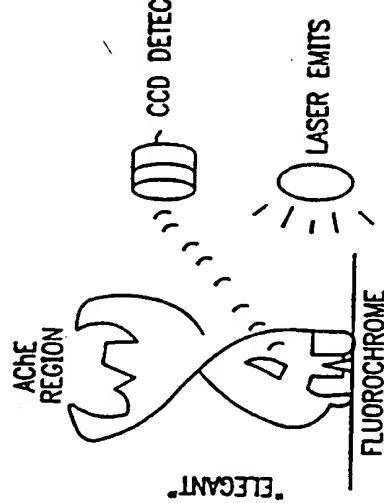
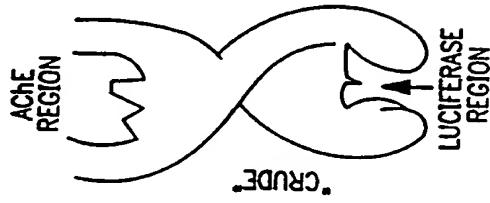
MICROPORT RELEASES EPITHELIAL GROWTH FACTORS + NERVE GROWTH FACTORS

**FIG. 17**

SILICON SYNAPSE

DETAIL VIEW OF ACETYLCHOLINESTERASE-FLUORESCENT FUSION PROTEIN

PURPOSE: FOR THE NEURONAL AXON TO TRANSMIT A SIGNAL TO THE SILICON SYNAPSE THE NERVE MUST RELEASE ACETYLCHOLINE IN THE USUAL MANNER. THE ACETYLCHOLINE MUST BE IN CLOSE PROXIMITY TO THE FUSION PROTEIN. KEEPING THE NEURON ASSOCIATED MAY BE PRODUCED BY RELEASE OF GROWTH HORMONES SLOWLY INTO THE AREA VIA A MICROPORT. ALSO AN ELECTRODE IS NEARBY ALSO CAUSING THE NEURON TO "FEEL" THE ARTIFICIAL SYNAPSE, BY THE INPUT OF WEAK+SMALL ELECTRIC CURRENTS. THERE ARE 2 VERSIONS OF THIS SYNAPSE, ONE "CRUDE" THAT DOES NOT HAVE A LASER, ONLY A CCD AND A LUCIFERIN PORT. THE "ELEGANT" USES A LASER TO EXCITE THE FUSION PROTEIN TO FLUORESCCE IF ACETYLCHOLINE IS PRESENT OR THE CONVERSE.



SPECIFICS OF FUSION PROTEIN

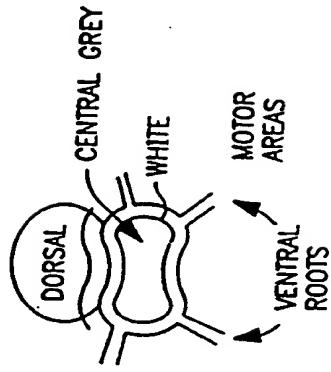
- (1) CONFORMATIONAL CHANGE TO BECOME ACTIVE WHEN ACh IS PRESENT
- (2) NON-ANTIGENIC
- (3) STABLE PROTEIN SO IT DOES NOT HAVE TO BE CHANGED

FIG. 18

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PLACEMENT OF SILICON SYNAPSE ELECTRODES

SPINAL CROSS-SECTION



- (1) PLACEMENT OF ELECTRODES INTO THE CORRECT STEROTAXIC LOCATIONS CAN BE ACHIEVED BY MRI LOCALIZATION.
- (2) LASER MICROHOLLES CAN BE DRILLED INTO THE SPINAL CORD WITH SUITABLE CO₂ OR OTHER LASER
- (3) PLACEMENT WOULD BE FROM SUPERFICIAL TO DEEP ALONG THE KNOWN PATHWAYS AND THERE COULD BE SEVERAL WIRES LEADING TO THE COMPUTER. RT/LT/LAT → MED/VENT → DORSAL AT FIRST 2 CHANNELS ON EACH SIDE BUT MANY MORE COULD BE PLACED
- (4) MUSCLE MOVEMENTS COULD BE INITIATED BY INSERTION OF PERMANENT ELECTRODES INTO VARIOUS MUSCLE BUNDLES
- (5) THE PATIENT WILL CONTROL THE OUTPUT BY THINKING ABOUT IT AND THEREBY RELEARNING MOTOR SKILLS, SUCH AS WALKING

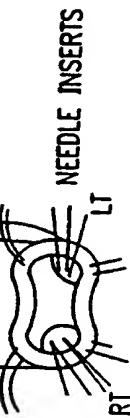


FIG. 19

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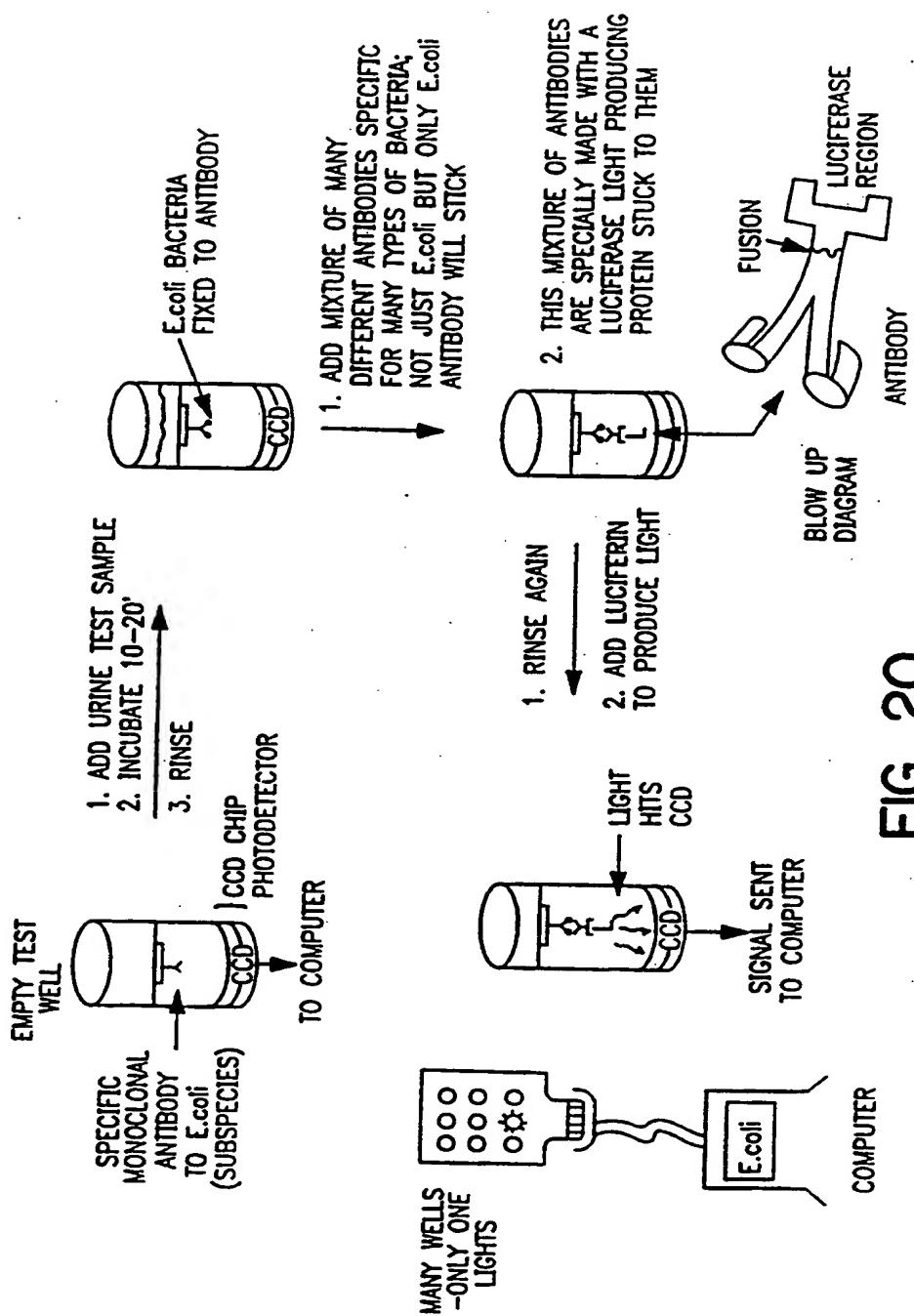


FIG. 20